Microencapsulation of *Bifidobacterium animalis* and *Lactobacillus casei* using resistant starch from *Vigna unguiculata*

Submitted in complete fulfillment for the Degree of Master of Applied Sciences (Food Science and Technology) in the Department of Biotechnology and Food Science, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa

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Reference Declaration

I, Ms. Danielle Rengadu - 21407659 and Prof. John Jason Mellem do hereby declare that in respect of the following dissertation – Title: **Microencapsulation of *Bifidobacterium animalis* and *Lactobacillus casei* using resistant starch from *Vigna unguiculata***

1. As far as we ascertain:

   a) no other similar dissertation exists;
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This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Prof. John Jason Mellem.**

Student's signature

To my Lord and Saviour **Jesus Christ,**

through him, I can do all things.

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Publications

Publications:

- Rengadu, D., Gerrano, A.S. and Mellem, J.J., **2020**. Prebiotic effect of resistant starch from *Vigna unguiculata* (L.) Walp. (cowpea) using an *in vitro* simulated digestion model. *International Journal of Food Science & Technology*, *55*(1), pp.332-339.

- Rengadu, D., Gerrano, A.S. and Mellem, J.J., **2020**. Physicochemical and structural characterization of resistant starch isolated from *Vigna unguiculata*. *International Journal of Biological Macromolecules*, *147*(1), pp.268-275.

Preface

The following dissertation is organized into five chapters and is presented as follows:

Chapter 1:

Introduction (describes problem statement, aims and contribution to knowledge relating to research).

Chapter 2:

Literature review (review of previous related studies and potential knowledge gaps.

Chapter 3 (Research objective 1):

Physicochemical and structural characterization of resistant starch isolated from *Vigna unguiculata*

Chapter 4 (Research objective 2):

Microencapsulation of *Lactobacillus casei* and *Bifidobacterium animalis* using resistant starch from *Vigna unguiculata*

Chapter 5:

Summary and conclusions (general discussion of key research findings, limitations, recommendations and future work).

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Abstract

The use of functional foods is increasing globally with individuals aiming to maintain a healthy gut causing an increasing trend associated with probiotics in the health sector. Probiotics are live microorganisms that aid in improving the digestive system and gut health, however, the main problem associated with probiotics are ensuring a safe delivery through transition to the colon in harsh gastrointestinal conditions. For probiotics to be considered effective to the host a growth of Log 7 is essential in the colon, thus the need for microencapsulation. Therefore, this study was aimed at analysing resistant starch isolated from cowpea as an encapsulation material for *Lactobacillus casei* and *Bifidobacterium animalis*, for beverage application. Five different cultivars of cowpea (*Bechuana white*, *Fahari*, *PAN 311*, *TVU 11424* and *DT129-4*) were analysed to determine the amount of resistant starch yielded as well as structural and physicochemical properties to determine the most suitable cultivar for the encapsulation process. The resistant starch percentage obtained was found in the range of 9.42-13.74%, with *DT129-4* yielding the most resistant starch. The structural and physicochemical results obtained showed that the resistant starch isolated from cowpea has the potential for microencapsulation with cultivar *DT129-4* exhibiting the most favourable results. Resistant starch was used as an encapsulating medium for *Lactobacillus casei* (RSL), *Bifidobacterium animalis* (RSB) and for a combination of the two probiotic microorganisms (RSLB) at a ratio of 1:1. The encapsulation yield after freeze drying were between 81.55-88.78% with the viability of the microcapsules under simulated gastrointestinal conditions also observed. The microcapsules were added to apple juice and the viability and stability of the microcapsules examined over 28 d. The final viability for microcapsules in the juice at the end of 28 d for RSL, RSB and RSLB were 7.53, 6.98 and 7.46 Log CFU/mL. This study shows that that resistant starch from cowpea has great potential as an encapsulating membrane within the nutraceutical beverage manufacturing industry.

Chapter 1: Introduction

Currently health conditions have become a major concern among consumers, who are giving more thought to the types of food that they are consuming and the associated health benefits. With an increase in gastrointestinal diseases, there has been an increase in the interest of probiotic-based foods that aids in digestion and various other health benefits (Fuentes-Zaragoza et al., 2010). Probiotics are known to be live microbial organisms that are considered beneficial to the host. These organisms have a wide range of health benefit such as the maintenance of a healthy colon by improving digestion and nutrient absorption, and exhibiting anti-carcinogenic, anti-infection, and antimutagenic activity (Das et al., 2014, Lam, 2017). However, one of the main problems associated with these probiotic-based products is their low survival rate within the food product as well as the gastrointestinal tract (Nazzaro et al., 2012). Viability of probiotic bacteria at the point of consumption and during consumption is important as they are affected by processing during manufacturing and transition during the gastrointestinal tract. Microorganisms such as *Lactobacillus* and *Bifidobacteria* species are commonly used as probiotics (Das et al., 2014).

Studies have shown microencapsulation to be one of the efficient ways to protect the probiotic microorganism (Nazzaro et al., 2012). Microencapsulation is a process whereby live cells are retained within an encapsulating membrane (Sultana et al., 2000). This method maintains viability and assists with appropriate release of probiotics at a specific point in the gastrointestinal tract where the host receives the full associated benefits. Benefits of microencapsulation include protection from bacteriophages, increasing survival during freeze-drying, freezing, storage over long periods and transition in the gastrointestinal tract (Das et al., 2014). There are diverse types of microencapsulation methodologies such as emulsion, extrusion, spray drying, gel-particle technologies as well as spray-chilling and adhesion to starch (Li et al., 2009). Some of the common materials used for microencapsulation of *Lactobacillus* and *Bifidobacteria* are polysaccharides such as alginate, gelatine, carrageenan, chitosan and starch (de la Cruz Pech-Canul et al., 2020). There is a growing interest in the use of prebiotics as an encapsulating membrane during microencapsulation (Martin et al., 2015). Resistant starch is considered as a prebiotic which are parts of food ingredients that are non-digestible and promotes the growth of good bacteria in the colon (Zaman and Sarbini, 2014).

The use of resistant starch in the pharmaceutical and food industry is continuously growing. A study done by Englyst et al. (1992) states that the sum of starch that resists digestion within the small intestine is known as resistant starch. This form of starch can be used as an ingredient, in food products that have a significant amount of dietary fibre, has been implicated in the improvement of digestive health as well as aiding in other positive biological effects such as nutrient uptake and synthesis of vitamins (Fuentes-Zaragoza et al., 2010, Haugabrooks, 2013). Potential physiological effects of resistant starch are the improvement of bowel health and acting as a prebiotic and culture protagonist (Nugent, 2005). Resistant starch can withstand enzymatic degradation as well as acidic degradation, therefore, making it an appropriate material for encapsulation. However, there are many factors such as physical and functional properties to be taken into consideration before the utilization of an ingredient in food applications. Physical characteristics include morphological as well as functional properties, analysis of these characteristics may determine if resistant starch is suitable for food application. Therefore, the study below analyses the physicochemical properties of resistant starch isolated from *Vigna unguiculata* (Cowpeas) and the potential use of resistant starch as a wall material for microencapsulation of *Lactobacillus casei* and *Bifidobacterium animalis*. Cowpea is a grain legume native to Africa. It is a cheap, readily available and abundant grain legume in Africa and has many possible food applications which can potentially help to curb food insecurity in many developing countries in Africa (Oyeyinka et al., 2020). Cowpeas are highly nutritious and are a good source of proteins, fats , vitamins, minerals, carbohydrates as well as resistant starch (Naiker et al., 2019, Oyeyinka et al., 2020).

Chapter 2: Literature Review

2.1. Gut microbiota and human health

Microbiota is the collective bacteria that is found on the mucosal surface of an individual, most mammals are born without these organisms and immediately after birth colonization takes place in the gastrointestinal tract. These microorganisms are anaerobic, facultative anaerobes and aerobic and are of extreme importance in developing specific gastrointestinal functions. There are more than 400 species of microorganisms present in the gastrointestinal tract with the proportion of anaerobic microorganisms increasing from the proximal to the distal regions of the gastrointestinal tract (Servin, 2004). The density, diversity and activity of gut microbiota affect the human health, the homeostasis of the gastrointestinal environment and the ability to cause disease. There are several internal and external factors influencing the gut microbiota's behaviour, composition and density. Diet, disease, lifestyle and living environment contribute to external factors, while physiological processes, genetics, age, stress, and the physiology and structure of the digestive tract collectively contribute to internal factors (Clemente et al., 2012).

There are certain gut microbiota that may affect the gut and cause dysbiosis resulting in allergies, inflammatory bowel disease, obesity as well as cancer (Macfarlane et al., 2009, Clemente et al., 2012). In the stomach, *Helicobacter pylori* are present at 10^3 cells/mL, while the small intestine plays host to *Lactobacilli* and gram-positive cocci ranging between 10^4-10^6 cells/g. The large intestine is home to a variety of microbiota e.g. *Bifidobacteria, Lactobacilli, Bacteroides, Enterobacteriaceae, Enterococci* and *E. coli* with these bacteria playing a major role in metabolic activity as well as producing short-chained fatty acids (Clemente *et al.*, 2012). The colon is the human body's most complex and metabolically active organ, with characteristics of the colonic environment making it suitable for the growth of microorganisms. The ecosystem has readily available nutrients, a favourable pH and a slow transit time, supporting bacterial growth. Firmicutes and Bacteroidetes are the main bacteria involved in the metabolism of undigested food contaminants. The main type of bacteria present in the colon is anaerobic bacteria such as *Escherichia* sp., *Lactobacillus* sp., *Bifidobacteria*, *Enterococcus* sp., as well as *Streptococcus* sp. (Canny and McCormick, 2008). Some gut microbiota may have beneficial health effects whereas others are detrimental to health, with bacteria that have exclusive saccharolytic metabolism considered to be beneficial.

The composition of gut microbiota which affects health and disease are influenced by the physiological conditions that affect the host as well as environmental circumstances (Brownawell *et al*., 2012). These are considered as symbiotic bacteria and contribute to the gut defence system through homeostasis of the immune system. This relationship maintains the colonic environment, regulates gut motility, synthesizes vitamins and absorbing minerals, transforms bile acids as well as activating and destroying toxins present. They also play a role in preventing harmful bacteria from growing and stimulating the immune system (Morrison and Preston, 2016). Examples of microbiota associated with harmful or negative effects are *Staphylococci*, *Proteus* and *Clostridia* which display the following adverse effects in the host: diarrhoea and or constipation, gastrointestinal infections, liver damage, cancer as well as produces pathogens and carcinogens and aids in intestinal putrefaction (Gibson et al., 2010, Patel et al., 2014). Bacteria which are beneficial to the host are *Lactobacillus*, *Eubacteria*, and *Bifidobacterium* which assists in the synthesis of vitamins and minerals, stimulates the immune system and aids in digestion and absorption of food. Bacteria that have the potential to display both beneficial and adverse effects are *Escherichia*, *Enterococci*, *Streptococci*, and *Bacteroides* (Morrison and Preston, 2016).

Gastrointestinal diseases occur due to the effect of certain gut microbiota on specific parts of the gastrointestinal tract. These microbiotas play an important role in the adverse effects that are experienced in most common diseases such as inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS) with symptoms of constipation and diarrhoea. Pathogenic organisms proliferate the epithelial surface of the intestine and invade the underlying mucosa. One of the first microorganism that has been implicated in causing IBD is *E. coli*. Studies have shown to have increased levels of *E. coli* in bowel tissues and are more adhesive to epithelial cells of people suffering from IBD as opposed to healthy people (Macfarlane et al., 2009). The symptoms are as follows: nausea and vomiting, abdominal pains, belching, bloating and flatulence as well as chronic diarrhea, etc. (Macfarlane et al., 2009). The above diseases have severe effects on human health; however, these gastrointestinal diseases may be due to poor lifestyle and dietary habits. Changes in one's diet can have positive potential benefits on the health of the host through breakdown and fermentation of dietary fibre and resistant starch, which then produce fatty acids that have immune-modulatory effects (Morrison and Preston, 2016, Ma et al., 2017).

In the gastrointestinal system, healthy microbiota play an extremely important role and contributes to beneficial health properties which include protection against infectious diseases, synthesis, and uptake of nutrients and improving the immune system (Birt *et al.*, 2013). Gut bacteria also regulates gut motility, transform bile acids and steroids and destroys toxins (Clemente *et al.*, 2012). Prebiotics have the potential to prevent and treat various diseases due to the relationship between the gut microbiota and host (Birt *et al.*, 2013).

2.2. Prebiotics and Probiotics

Prebiotics and probiotics are considered functional food ingredients. These are non-digestible food ingredients that have benefits beyond basic nutrition and are also considered to be nutraceuticals. A nutraceutical can be characterized as a non-toxic food ingredient with clinically proven health benefits associated with disease prevention and treatment (Shinde et al., 2014). Probiotics, as well as prebiotics, are used to promote a healthy gut however probiotics work well in the short term upon regular use. Since they can promote the development of beneficial bacteria in the colon, probiotics are used in dietary supplements, thereby enhancing digestion and absorption of nutrients. According to Gibson and Roberfroid (1995) a probiotic has to meet the following criteria:

i. Can be prepared on a large scale on a viable basis and remain viable through processing and use
ii. Survive intestinal ecosystem and benefit the host

Prebiotics, on the other hand, are considered to have long-term effects upon regular intake, with the consumption of foods containing resistant starch helping to improve and maintain the natural microflora within the colon (Lam, 2017). According to Macfarlane and Cummings (1999), a prebiotic has to meet the following criteria:

i. Be able to undergo fermentation by microorganisms
ii. Promote the growth of microbiota considered useful to the host

Prebiotics may extend beneficial properties beyond the stimulation of *Bifidobacteria* and *Lactobacillus* and may recognise health benefits from other beneficial microorganisms such eubacteria. According to Gibson et al. (2017), prebiotics depend on microbial metabolism as fermentation of the prebiotic by microorganisms, producing fatty acids that can be potentially beneficial to the digestive system.

Substances such as polyunsaturated fatty acids are converted to short-chained fatty acids that are like the product of prebiotic fermentation. Inulin, fructo-oligosaccharides and polydextrose are examples of proven prebiotics, while yeast, *Lactobacilli*, *Bifidobacteria* and other beneficial bacterial strains are examples of probiotics (Gibson et al., 2017, Sarao and Arora, 2017).

2.2.1. Prebiotics

Prebiotics, well-known functional foods, are additives in non-digestible foods that promote the development of one or more beneficial colon bacteria. Prebiotics are found as relatively short chains of carbohydrates. To benefit human health, resistant starch and lactulose have been used generally in the food industry to modify the composition of the microbiota species, since prebiotics have the potential to change the genetic makeup of the gut microbiota (Zhang et al., 2015). Prebiotics have potential benefits in maintaining a diverse gut microbiota together with regulation of the immune system as well as the production of bacterial metabolites. They also increase the growth of specific gut microbiota such as *Bifidobacteria* and *Lactobacillus*, this plays an important role in alleviating lactose intolerance (Patel et al., 2014, Wilson and Whelan, 2016).

After extensive studies, fructose-oligomers, and galactooligosaccharides have been granted a prebiotic status (Roberta Grimaldi, 2016, Gibson et al., 2017). Both have been proven to stimulate the growth of *Bifidobacteria,* which produces metabolites such a short-chained fatty acid which can later benefit the host (Zaman and Sarbini, 2014). Prebiotics also occur naturally in many fruit and vegetables such as garlic, bananas, onion, and wheat (Zhang et al., 2015). Diets that are rich in prebiotics have many beneficial effects on the host such as regulation of the gastrointestinal tract and production of propionate, acetate, butyrate which are short-chained fatty acids and other essential fatty acids, stimulates and regulates the immune system, reduces the risks of chronic disease and helps treat certain diseases and disorders (Thavarajah et al., 2016). According to Sarao and Arora (2017), useful prebiotics exhibit the following criteria:

- Required in low dosages
- Low in calories
- Able to easily be incorporated in food without drastically changing the properties of the final product
- Act as a good preservative
- Must be able to target the large intestine

2.2.2. Probiotics

In the early nineteenth century the scientist, Ilya Mechnikov founded the concept of probiotics by proposing the ingestion of lactic acid bacteria to aid human health (Vaughan et al., 2005). Another scientist, Henri Tissier, discovered *Bifidobacteria* and suggested that they be used as infant probiotics (Vaughan et al., 2005). Most probiotic based food products contain strains of *Bifidobacteria* as well as lactic acid bacteria (LAB). There is a great interest in how probiotic bacteria work to improve the human gut, with physiological benefits, interactions and mechanisms of these probiotic bacteria widely researched in academia, food industry and pharmacology (Vaughan et al., 2005). Probiotics are live bacterial feed nutrients with beneficial effects on the host such as helping improve the gastrointestinal tract and assisting with digestion. Probiotics must have the ability to survive the difficult change within the gastrointestinal tract such as enzymatic or acidic environments. It must be able to reach the large intestine in sufficient amounts and be able to colonise and proliferate within the large intestine (Shori, 2017). Different types of bacteria, yeast and mould have the potential to display probiotic effects. Some of the most common species of bacteria are *Lactobacillus, Bifidobacterium, Bacillus, Streptococcus, Propionibacterium and Enterococcus*. Some of the common yeast and moulds are *Candida pintolopesii, Aspergillus niger Saccharomyces cerevisiae, Aspergillus oryzue and Saccharomyces bourlardii* (Amara and Shibl, 2015).

Bifidobacteria and *Lactobacillus* are both gram-positive, facultatively anaerobic, non-spore-forming and produces lactic acid. The amount of *Bifidobacteria* and *Lactobacillus* present in the human gut fluctuates during their lifespan. The composition within the GIT is affected by microbial and environmental factors. There are various species and strain-specific regions in which they can adapt to different lifestyles in these probiotic microorganisms, *Lactobacillus* thrives in environments which are nutritionally rich and can ferment sugars and rapidly convert them to lactic acid (Papizadeh et al., 2017). *Bifidobacteria* are important as they produce short-chained fatty acids upon fermentation.

Due to many factors, such as improper nutrition, drugs, age, etc., their volume could be degraded, hence the need for our regular diet to include them. Probiotics have the potential to be anti-mutagenic and anti-carcinogenic, stimulate the immune system, cholesterol reduction as well as alleviating the symptoms and effects of lactose intolerance and nutritional enhancement (Das et al., 2014). Probiotics have functions in the growth and division of epithelial cells, as well as immune system production, homeostasis and can contend with the poor microbes in the gastrointestinal tract.

Some of the ideal characteristics of probiotic strains are (Pandey et al., 2015):

- Non-pathogenic
- Produces lactic acid
- Acid and bile tolerant
- Genetically stable
- Effective adhesion to the gut lining
- Anti-genotoxic properties
- Short time generation
- Robust and surviving processing conditions

Criteria for probiotics fall under three categories which are safety, functional and technological. According to Mitropoulou et al. (2013) requirements are as follows:

i. Safety - the probiotic needs to be a non-pathogenic bacterium which is of human origin and is generally regarded as safe.
ii. Functional - the probiotic must be able to attach to the epithelial wall of the intestines, to be capable of colonising the colon, influence metabolic activities as well as modulate the immune response
iii. Technological - the probiotic must be resistant to certain technological processes as well as the ability to survive processes that are scaled up.

Probiotic bacteria such as Lactic acid bacteria and *Bifidobacteria* are usually associated with the GIT and can suppress the growth of pathogenic bacteria which cause gastrointestinal diseases and disorders as well as stabilising the digestive system by creating and increasing the intestinal barrier function (Das et al., 2014). Due to current research trends, there is an increase in the microorganism that can be classified as probiotics as certain strains of Bacillus, Pediococcus, as well as some yeasts has been reported as promising probiotic microorganisms (Yao et al., 2020). Probiotics can be used as a supplement and can be found in traditional foods such as yoghurt, cheese and beverages. Probiotics can be used for fermentation of different foods to develop simple beneficial by-products. To ensure that the minimum requirement of 10^6-10^7 colony-forming units per gram (CFU/g) could reach the colon, probiotic-based food should be in the range of 10^8-10^9 CFU/g before ingestion to confer potential health benefits of the associated probiotics (Chew et al., 2019, Yao et al., 2020).

Survival of probiotics is dependent on many factors, most probiotics are not able to survive in low pH of the gastric juice present in the stomach and exposure to oxygen limits their viability and stability in most food products (Shori, 2017). Microencapsulation is one of the efficient ways to protect the probiotic microorganism and contributes to developing various functional foods as it retains and protects the probiotic bacteria in food products and through the digestive system (Nazzaro et al., 2012). Cells are incorporated into an encapsulating membrane which shields them against deterioration due to harmful environmental factors and controlled release at regulated levels under specific conditions (Shori, 2017).

2.3. Microencapsulation

Chemical, thermal and enzymatic processing are commonly used processing methods in the food industry and these methods can have a negative effect on valuable nutrients and microorganisms in food products. Therefore, there is a necessity to have a protective coating material or shield to ensure that the valuable attributes of the food product remain, to ensure safe delivery into the human system. This technique is known as microencapsulation (Panghal et al., 2019).Microencapsulation is a process whereby bioactive compounds in the form of gas bubbles, liquid droplets, solid particles or even live cells are retained within an encapsulating membrane and is widely used in the food industry and pharmaceutical industry (Zhang et al., 2018). A variety of food ingredients and products would have not been made possible without the use of microencapsulation technology, as it has impacted the food industry by incorporating different molecules and cells such as enzymes, additives, probiotic bacteria, flavours, fragrant, antioxidants, nucleic acids, vitamins as well as minerals into capsules to provide stability and protection in various environments (Liu et al., 2019, de la Cruz Pech-Canul et al., 2020). Most technologies for microencapsulation use a liquid or gas medium(de la Cruz Pech-Canul et al., 2020). The main purpose of encapsulation is the protection of the core functional ingredient, with regards to probiotic encapsulation, one of the most important factors is the type of wall material to be used. The most widely used wall materials for probiotic microencapsulation is polysaccharides and proteins (Liu et al., 2020).

According to de la Cruz Pech-Canul et al. (2020) and Yao et al. (2020), there is a wide range of benefits associated with microencapsulation such as:

- Protection in harsh and unstable environmental conditions
- They serve as a protection against bacteriophages
- Immobilises microorganisms and enzymes
- Multiple delivery systems of nanoparticles
- Increases stability and viability during processing
- Improves solubility as well as dispersion and flow
- Increase shelf life and storage
- Increase survival during freezing and freeze-drying
- Protects and increases the stability and viability of components during the transition in the gastrointestinal tract
- Allows for the controlled release of functional components

Delivery systems release may be triggered by the change in pH and ionic strength, enzymatic activity such as the use of a catalyst, heating, shearing and solubilization (Das et al., 2014). Microencapsulation usually uses different materials e.g., water-based and oil-based material to allow multiple delivery systems. For example a water-oil- water emulsion is obtained with small water droplets which are dispersed into larger oil droplets which are dispersed in a larger water droplet, therefore, maintaining multiple delivery systems whereby the microencapsulated components can serve different functions (Nazzaro et al., 2012).

It is of utmost importance to choose the appropriate encapsulating membrane with regards to stability as well as safety. Safety of the delivery system is highly important when live cells are being encapsulated for the oral delivery system to the gastrointestinal tract because the live cells must be protected during encapsulation and the entire delivery system should not disintegrate in harsh environmental conditions. Safety ensures the survival of cells during the transition in the gastrointestinal tract (Afzaal et al., 2020). The membrane material used must block out harmful or toxic molecules as well as simultaneously provide permeation for nutrients to pass through. Most membrane materials are natural ingredients which are considered GRAS (Generally Recognised as Safe). The most appropriate membrane material is cost-efficient, readily or easily available, biocompatible, non-toxic or harmful and easy to handle (Ramos et al., 2018). Various food companies in the industry rely on microencapsulation technology to manufacture products such as the beverage, dairy, baking and meat industry.

These industries continue to create novel ingredients and products such as functional ingredients, anti-oxidants, various colourants and flavours as well as vitamin and mineral enriched products (Peanparkdee et al., 2016). In a study done by Seyedain-Ardabili et al. (2016) probiotic cultures, *Lactobacillus acidophilus* LA-5 and *L. casei* 431, were encapsulated using calcium alginate and Hi-Maize resistant starch as the membrane material which was coated with chitosan. These microcapsules were inserted into bread to produce symbiotic bread. The bread produced met the criteria for probiotic products. In another study done by Burin et al. (2011) anthocyanins were encapsulated in different encapsulating membranes in a soft drink. The purpose of the anthocyanin was for the colour of the soft drink, however, anthocyanins are unstable and due to change in pH, temperature and oxygen they can be reduced to a colourless compound. Therefore, to allow for the effectiveness of the anthocyanin, encapsulation took place. Several factors affect microencapsulation such as (Kavitake et al., 2018):

- Environmental conditions
- Encapsulating membrane material and modifications
- The conditions of processing and techniques used for encapsulation
- The concentration of the polymer and bead diameter
- Characteristic's o the capsule in different environments
- Effect of the bacterial cell on the microcapsules

These factors may be overcome by selecting the appropriate membrane material, selecting the correct encapsulating technique and considering the environmental conditions. According to Peanparkdee et al. (2016), there are many challenges associated with microencapsulation. The selection of the microencapsulation technique is extremely important, the technique and the methodology must be most suitable for the product being encapsulated i.e. freeze-drying will be most suitable for heat-sensitive materials (Liu et al., 2020). In a study by Coghetto et al. (2016), *Lactobacillus plantarum* was encapsulated in sodium alginate using electrospraying and the results were found to increase the survival rate of the probiotic. Another study by Ranadheera et al. (2015) encapsulated probiotic bacteria using spray drying methods, while Zanjani et al. (2014) encapsulated *Lactobacillus casei* and *Bifidobacterium bifidum* using calcium alginate-gelatinized starch with a chitosan coating and inulin using the emulsion technique. The microcapsules were subjected to *in vitro* digestibility and results have shown that encapsulation has increased the rate of survival of the probiotic.

2.3.1. Common microencapsulation techniques

There is a wide range of techniques that may be used for microencapsulation which fall under three categories i.e., physical, chemical and physicochemical. The microencapsulation technique used is dependent on various factors such as the properties of functional ingredient and the core material as well as the required particle size and release mechanism of microcapsules (Arredondo-Ochoa et al., 2018, de la Cruz Pech-Canul et al., 2020). As shown in Figure 2.1 the technique used for microencapsulation affects the diameter size of the microcapsules.

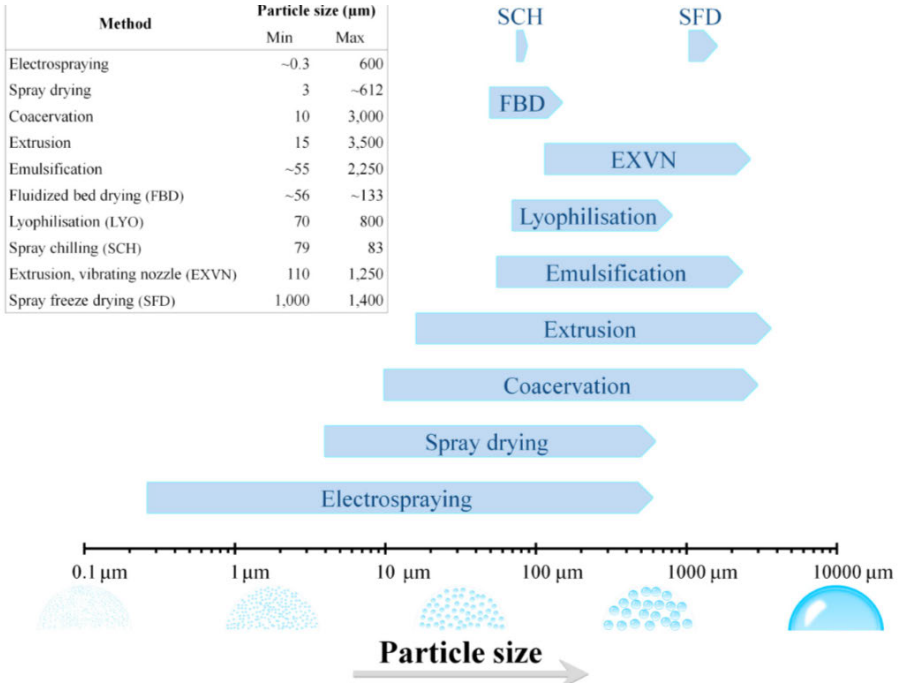

Figure 2.1: The effect of encapsulation technique on particle size (de la Cruz Pech-Canul et al., 2020).

The most commonly used techniques are spray drying, spray cooling, simple extrusion, ionic gelation, fluidized bed coating, and emulsification (Arredondo-Ochoa et al., 2018, Chew et al., 2019). Extrusion is one of the most popular techniques used for microencapsulation of probiotics on a laboratory scale, due to its simplicity, affordability and effectiveness to increase protection of functional ingredients. This method is done by adding the core ingredient into a hydrocolloid solution, the cell suspension undergoes extrusion through a syringe into hardening solution such as calcium chloride (Chew et al., 2019). The common wall material used for extrusion is polysaccharides. Despite the affordability of using the extrusion technique, the disadvantage of this method is the slow rate of solidification of microcapsules (de la Cruz Pech-Canul et al., 2020).

Emulsification is another cheap and easy method that uses a hydrocolloid solution. This is done by adding the core ingredient and the wall material in aqueous and oil phases, which forms small droplets. The mixture is homogenized to form a water-in-oil emulsion. With the addition of cross-linking agents such as calcium chloride, gel particles are formed within the oil phase. The microcapsules formed can be recovered by filtration or centrifugation.

The particle size of microcapsules is based on the rate of agitation, however, emulsifying agents such as tween can be added to decrease the interfacial tension, allowing better homogenization and the formation of smaller capsules (Raddatz et al., 2020, de la Cruz Pech-Canul et al., 2020). The main advantage is that emulsification can produce capsules smaller than 100 µm to as small as 2-25 µm and the disadvantages are the low yields of microcapsules and a high size variation (Ramos et al., 2018).

Coacervation is the separation of colloidal systems into two liquid phases. Factors such as pH, ionic strength, polarity and temperature influences coacervation. There are two types of coacervation i.e. simple which is when a single macromolecule is present and complex where two or more macromolecules are present (Eghbal and Choudhary, 2018). The advantages of this method is that is cost-efficient, simple to conduct, does not require organic solvents or high temperatures (da Silva et al., 2014). The disadvantages of this method are the difficulty in producing microcapsules with small sizes, the cost and the complexity of the process (Ramos et al., 2018). However, the main disadvantage is that coacervation can only occur within a limited pH range and limited colloid and electrolyte concentrations (Bratovcic and Suljagic, 2019).

Spray drying is another common method used in the food and pharmaceutical industry. The primary solution for spray drying consists of the core ingredient and the wall materials, therefore the wall material must be soluble in water. According to Chew et al. (2019), since the core material reaches the lower temperature, spray drying may be used in heat-labile materials. The mechanism of spray drying involves the dispersion of probiotic cells into an aqueous solution of the wall material, then atomization of the solution occurs through a nozzle, evaporation takes place when the solution comes into contact with hot air, the dried particles are then separated from the hot air and is collected with the aid of a cyclone (Frakolaki et al., 2020). A primary solution with a low viscosity is more desirable than that with a high viscosity to prevent clogging and build up in the pipes and nozzles and to allow for uniform spraying (Ushiyama and Shimizu, 2018).

Factors that affect the outcome of the spray-dried capsules are dependent on the probiotic and wall material characteristics, drying time, inlet and outlet temperature, the pressure and the diameter of the nozzle.

Spray drying is rapid, cost-efficient and easy to use on a large scale as it only uses one unit of equipment (Frakolaki et al., 2020). It can protect the core ingredient but due to the high temperatures required to ensure water evaporation the viability of the probiotic is reduced. However, this can be overcome by using prebiotics (Sarao and Arora, 2017, Frakolaki et al., 2020)

According to de la Cruz Pech-Canul et al. (2020), spray drying and spray cooling are similar procedures as both involves atomization of the core materials into a chamber that forms the microcapsules through the appropriate environmental condition. The dried microcapsules are separated and are collected by a cyclone. The environmental conditions are the main difference between these methods. Spray drying uses high temperatures while spray chilling uses lower temperatures. Spray chilling occurs when the hot mixture of the solution undergoes atomization in temperatures which are lower the melting point of the wall material.

Freeze drying is another method of encapsulation, it is mostly used for heat-sensitive components such as microorganisms and involves the freezing and sublimation of water from the solid-state directly to a gas state under a vacuum (de la Cruz Pech-Canul et al., 2020, Frakolaki et al., 2020). In a study done by Tang et al. (2020) spray- drying and freeze-drying was used to encapsulate *Lactobacillus acidophilus* FTDC 3081, separately in wall material i.e. skim milk, sucrose, maltodextrin, and corn starch. From the results obtained the methods used increased the survival rate of the probiotic bacteria under different storage temperatures, with both spray-dried and freeze-dried maltodextrin capsules showed higher protection than the free cells.

2.3.2. Common wall materials used for microencapsulation

Edible coatings that are commonly used for encapsulation are polysaccharides such as; alginate, chitosan, chitin, starch, cellulose and carrageenan, proteins such as; soy protein, whey powder, gelatin and lipids such as; fats and waxes (de la Cruz Pech-Canul et al., 2020), which can be seen in Figure 2.2.

The membrane material used must block out harmful or toxic molecules as well as simultaneously provide permeation for nutrients to pass through (Martin et al., 2015). It is important to choose materials that are safe and non-toxic as well as material that does not have adverse effects during interaction with compounds that requires encapsulation. Materials that will be used should follow the appropriate requirements from the Food and Drug Administration and should be Generally Regarded As Safe (GRAS) (Martin et al., 2015). Proteins are also commonly used along with other encapsulating agents. Some proteins are considered ideal to be used for microencapsulation. Gelatin has been used in studies as an encapsulating agent. In a study done by Mathews (2017), lactobacillus species were encapsulated in gelatin and the results obtained showed that gelatin increased the viability of the probiotic bacteria.

Alginate, a polysaccharide derived from brown algae, is the most commonly used core material for microencapsulation. Alginate is generally regarded as safe and is used in many other food applications worldwide. It is non-toxic and can cross-link. It has a great affinity to metals such as calcium, zinc and barium. When combined with these metals, alginate forms greater protection and increases stability during microencapsulation. Cations like sodium may also be used together with alginate to strengthen the polymer matrix (Ramos et al., 2018).

However, a study conducted by Sultana et al. (2000) presented that one of the disadvantages associated with using alginate is that it may not completely survive the harsh environmental conditions in the gastrointestinal tract, therefore they are mixed with other starch polymers. Chitosan is a cation that may be used as a coating material with alginate as it helps reduce porosity. Like alginate, chitosan is also considered non-toxic and cost-effective to use. In a study done by Gandomi et al. (2016), encapsulation of *L. rhamnosus* improved viability as 27% of the probiotic population survived simulated gastrointestinal conditions. According to a study conducted by Shi et al. (2013), carrageenan-locust bean gum was used to encapsulate *L. bulgaricus* showed that the viability of was kept constant stored at 4˚C for one month

Recently prebiotics such as inulin, fructo-oligosaccharides as well as resistant starch has also been incorporated into the wall materials. The prebiotics increases the protection during gastrointestinal digestion due to their ability to survive harsh acidic and enzymatic conditions.

Valero-Cases and Frutos (2015) conducted a study on the use of different concentrations i.e., 0, 1 and 2% of inulin as a reinforcement with sodium alginate and xanthan gum for microencapsulation. The results obtained showed that there was an improvement of the survival of *L. plantarum*, with 2% of inulin as compared to the other concentrations. In other studies gum Arabic has been replaced with the use of modified starch combined with prebiotics such as inulin (Peanparkdee et al., 2016). According to a study conducted by Li et al. (2016), *L. plantarum* was microencapsulated in native maize starch or porous maize starches which protected the probiotic bacteria during simulated digestion, in another study done by Jantarathin et al. (2017) probiotics were encapsulated with prebiotics as well as alginate and chitosan which showed an improvement in the survival of cells during heat processing.

According to de la Cruz Pech-Canul et al. (2020), the wall material must have the following characteristics:

- Must be generally regarded as safe
- Be able to contain, seal and protect the core ingredients
- Be able to restrict the movement of the core ingredients
- Must be affordable and sustainable
- Must release core ingredients at specific points

Various studies showing the use of different probiotics, microencapsulation techniques and wall materials with the addition of prebiotics can be seen in table 2.1.

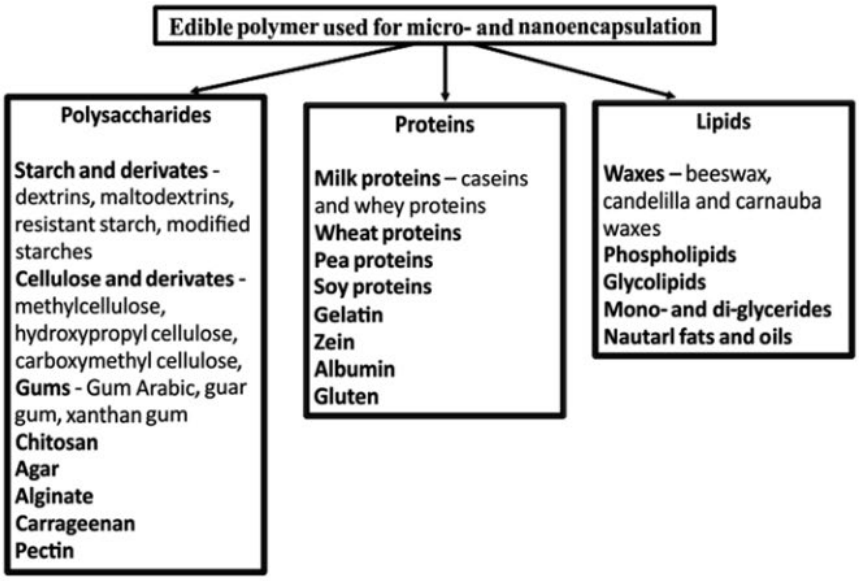

Figure 2.2: List of common wall materials for encapsulation (Arredondo-Ochoa et al., 2018)

Table 2.1: List of probiotics, wall materials with the addition of prebiotics and techniques used for microencapsulation

Probiotic	Wall material/s	Techniques	Reference
Bifidobacterium spp.	Modified waxy maize starch	Spray drying	(O'Riordan et al., 2001)
Lactobacillus casei, Bifidobacterium bifidum	Calcium- alginate, Hi-maize RS	Emulsion	(Fahimdanesh et al., 2012)
Lactobacillus acidophilus	Hi-maize RS, Chitosan	Extrusion	(de Araújo Etchepare et al., 2016b)
Lactobacillus acidophilus	Sodium alginate matrix, Inulin, Jerusalem artichoke, Chitosan	Freeze drying	(Jantarathin et al., 2017)
Lactobacillus casei LK-1	Skim milk, Trehalose, Maltodextrin	Spray drying	(Liao et al., 2017)
Lactobacillus acidophilus, Lactobacillus casei	WPI, FOS	Freeze drying	(Bora et al., 2018)
Lactobacillus rhamnosus ATCC 7469	WPI, WPI+ Inulin or WPI+inulin+Persian gum	Spray drying Freeze drying	(Moayyedia et al., 2018)
Lactobacillus acidophilus	Gum Arabic, inulin, hi-maize RS, Trehalose	Spray drying	(Nunes et al., 2018)
Lactobacillus plantarum, Weissela paramesenteroides, Enterococcus faecalis, Lactobacillus paraplantarum	Arrowroot starch, maltodextrin, WPI	Freeze drying	(Samedi and Charles, 2019)
Lactobacillus acidophillus	Sodium alginate, rice bran, Inulin, Hi-maize RS	Extrusion	(Poletto et al., 2019b)
Lactobacillus acidophilus	Sodium alginate with rice bran, inulin and resistant starch (Hi-maize)	External ionic gelation Freeze-drying	(Poletto et al., 2019a)
Lactobacillus acidophilus	Skim milk, Sucrose, Maltodextrin, Corn starch	Spray drying Freeze-drying	(Tang et al., 2020)

*RSM (Reconstituted skim milk); WPI (Whey protein isolate); FOS (Fructooligosaccharides)

2.4. Starch as a wall material for microencapsulation of probiotics

Carbohydrates are one of the main constituents of food and are a major source of energy. There are different classifications of carbohydrates ranging from simple to complex carbohydrates. Simple sugars also are known as monosaccharides are made up of one unit of sugar whereas complex carbohydrates consist of 2 or more units that are linked by glycosidic bonds which are disaccharides, oligosaccharides, and polysaccharides. Examples of polysaccharides include glycogen and starch (Haugabrooks, 2013). Starch is regarded as a main carbohydrate storage component in plants and is one of the major macronutrients constituents of foods. The interaction of starch with other food components such as protein, water and lipids are of great interest in the food industry (Wani et al., 2016).

Starch occurs naturally and is biodegradable, cheap and the most abundant polymeric biomolecules present in legumes. Amylopectin and amylose are the 2 polymers which make up starch. As shown in Figure 2.3 these polymers differ greatly in their properties and structure. Amylose has a compact linear structure whereas amylopectin is branched. When exposed to water, amylose molecules disperse and forms tough gels and strong films whereas amylopectin forms soft gels and weak films (Yu et al., 2014, Wani et al., 2016). The amylose content ranged between 17.00–51.69%. for various legume starches.

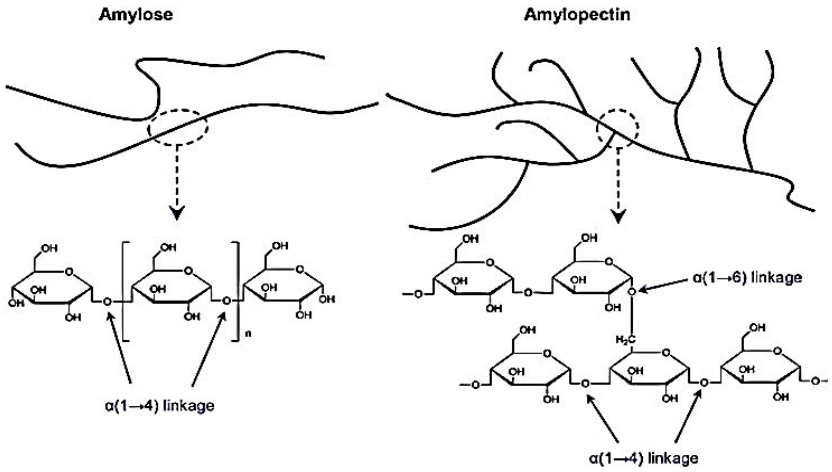

Figure 2.3: Structures for amylose and amylopectin of starch (Xie et al., 2014).

The nature of the crystallinity of various starch-based food is determined using X-ray diffraction. X-ray diffraction is used to differentiate between the types of crystallinity of starch. There are 3 types of crystallinity i.e. A-type, B-type and C-type. According to previous studies, starch granules have displayed a semi-crystalline character which indicates a high degree of orientation of glucan molecules. Amylose present within the starch displays an amorphous character whereas the amylopectin contributes to the crystalline region. Approximately 30% of the mass of starch granules is crystalline (Sajilata et al., 2006). Both polymers present in starch can form double helices which may react and form crystalline structures. Amylopectin is most common to form crystalline structures in starch (Tester et al., 2004). Type A is found in cereal, type B is found in roots and tubers and type C which is an intermediate between types A and B is present in legumes (Sandhu and Lim, 2008, Mahasukhonthachat, 2010). According to a study done by Ratnaningsih *et al*. (2016), X-ray diffraction patterns of cowpea starches showed strong C-type crystallinity.

Crystallinity is mostly dependent on 3 factors that are the chain lengths of the amylopectin, the density of the starch granules and the presence of water (Sajilata *et al.*, 2006). Type A usually consists of 23 to 29 chain lengths of amylopectin whereas type B has 30 to 44 chain lengths of amylopectin and type C having 26-29. The hydrogen bonding between each hydroxyl group contributes to the formation of the outer double helices. Crystallinity affects the hydrolysis of starch and the formation of resistant starch.

Starch exists as starch granules and the granule morphology varies amongst legumes and other sources of starch. Variation in granule morphology is observed using scanning electron microscopy (SEM). With SEM, granules have been reported to observe the characteristics of the starch granule i.e. shape, size, crevices, grooves and pinholes (Wani et al., 2016). The structural morphology of starch granules relies on the chloroplast and amyloplast's biochemistry and the plant's physiology (Ashogbon and Akintayo, 2013). According to a study done by Ashogbon and Akintayo, (2013) granules of cowpea starch appeared in clusters while some were singled. As shown in Figure 2.4 the starch granules present in cowpea are reported to be irregular as some were round, oval or kidney-shaped (Mwangwela et al., 2006).

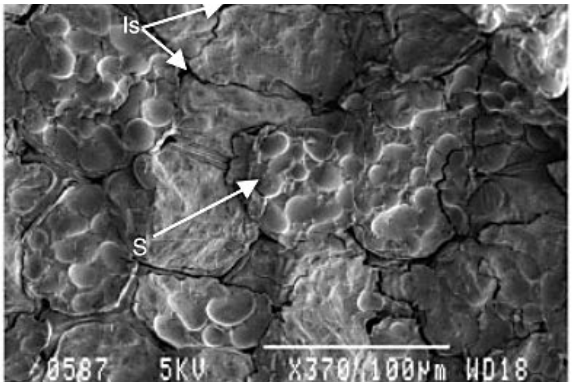

Figure 2.4: Scanning electron microscopy of the compact cotyledon structure of Bechuana white cowpeas, showing the starch granules/s and intercellular space/s (Mwangwela et al., 2006).

During thermal processing, starch granules are gelatinized due to the presence of excess water when native starch is cooked. Starch granules that undergo gelatinisation absorb water, swells and lose their crystallinity as well as their structure. The amylose and amylopectin chains become amorphous polymers. This forms a gel-like viscous paste however upon cooling the amylose and amylopectin starch chains and realign themselves and forms a semi-crystalline structure that is different from the native starch.

Retrograded starch is found in significant amounts in starchy food that has been heated and cooled. Functional properties of starch such as the swelling power and solubility index, syneresis, water absorption capacity and pasting may be affected by the difference in gelatinisation and retrogradation. The gelatinization temperature of most starches is between 60-80°C (Copeland et al., 2008, Wani et al., 2016). The proportion of crystallinity of starch granules affects the swelling power and solubility index. The starch granules swell upon heating and binding forces of the starch granule are weaken. An increase in swelling and solubility may be due to loose granule structure and low molecular weight of amylose. Many properties of starch may affect the swelling and solubility i.e., amylose/amylopectin ratio, chain length, branching as well as molecule weight. The rate of swelling is affected by the amount and composition of starch as well as the temperature and force (Copeland et al., 2008, Wani et al., 2016).

Syneresis is when the liquid in the gel formed through gelatinisation spontaneously oozes out from the gel. The water that is physically trapped in the gel is separated. Syneresis is a measurement of the degree of retrogradation in legume starch. Due to their higher amylose content as compared to cereal starch, legumes have a higher rate of syneresis (Wani et al., 2016). Water absorption activity refers to the amount of water held within the starch. Starch can hold water within the granules due to their hydrophilic molecular chains. The hydrophilic site allows for water interaction, therefore allowing water to be absorbed. Temperature correlates with water absorption as an increase in temperature increases water interaction (Adebooye and Singh, 2008, Wani et al., 2016). Pasting refers to changes in starch that occur upon further heating post-gelatinisation such as further swelling and solubility as well as a change in viscosity (Ratnaningsih et al., 2016).

Starch can be characterized into two types of starch which are digestible starch and non-digestible starch. The digestible starches are recognised as rapidly digestible starch (RDS) and slowly digestible starch (SDS), whereas the non-digestible starch known as resistant starch (RS) which escapes digestive process in the small intestine and finally reaches the colon (Englyst et al., 1992, Englyst et al., 1995). RDS are hydrolysed in the mouth and intestine by enzymes and acidic conditions in the stomach. RDS is digested at a rate of 20 minutes. In the small intestine, SDS is hydrolyzed by enzymes and has a digestion rate of 20-120 minutes (Lockyer and Nugent, 2017, Tacer-Caba and Nilufer-Erdilb, 2018). These fractions are measured during enzymatic hydrolysis of the starch. RDS is usually inversely proportional to SDS whereas as resistant starch is lower than both RDS and SDS (Raigond et al., 2014).

2.4.1. Resistant Starch

The portion of starch that resists hydrolysis and absorption in the mouth, stomach and small intestine is known as the resistant starch (Goni et al., 1997, Topping et al., 2003, Jyothsna and Hymavathi, 2017). Legumes and grains, raw potatoes as well as cooked and cooled potatoes, and food products with modified starch such as bread are some of the examples of resistant starch in food (Jyothsna and Hymavathi, 2017). The resistant starch stimulates the growth of natural beneficial microbiota present in the colon. The starch is fermented by the microbiota and produces short-chained fatty acids (Zaman and Sarbini, 2015, Yang et al., 2017). Resistant starch does not increase blood glucose with physiological effects like dietary fibre. Due to their low glycaemic index resistant starches are considered beneficial for health and aids in the prevention of insulin resistance diseases and beneficial to cardiovascular diseases (Jyothsna and Hymavathi, 2017). Resistant starch has been reported to reduce the risks of cardiovascular disease, circulating growth factors, reduce inflammation and reduce oxidative stress (Yang et al., 2017). It is either water-soluble or insoluble, that occurs naturally white and has a mild flavour (Erickson et al., 2018). According to Homayouni et al. (2014), resistant starch is a homo-polysaccharide that consists of several monosaccharide units linked together with α-(1–4) and α-(1–6) linkages. The starch consists of two parts amylose which is comprised of 500–600 glucan units and amylopectin with approximately 1000 glucan units. Various factors affect the formation of resistant starch such as gelatinisation and retrogradation as well as amylose content, chain length and crystallinity (Jagannadham et al., 2017).

2.4.2. Types of resistant starch

According to (Englyst et al., 1992) and (Englyst et al., 1995) resistant starch is classified into 3 types RS1-RS4, later on RS5 was added to the classification (Homayouni et al., 2014, Ashwar et al., 2016)

- RS1 is considered as the physically inaccessible starch that is entrapped within a non-digestible matrix, RS1 is usually found in legumes, seeds and grains (Zaman and Sarbini, 2015, Bello-Pereza et al., 2018). The cell walls of such food products are strong and can resist digestion, therefore, the starch present within the legume becomes inaccessible to degradation and hydrolysis. This type of resistant starch can be completely digested within the small intestine if it is properly refined (Lockyer and Nugent, 2017)

- RS2 is an ungelatinized granular starch that is present in raw potatoes and unripe banana and is known to resist digestion by α- amylase. RS2 has a high amylose content and due to its natural primary compact structure and the formation of starch granules (Lockyer and Nugent, 2017, Tacer-Caba and Nilufer-Erdilb, 2018).
- RS3 is non-granular retrograded starch which is the association of the starch molecules that can form a gel and is found in cooked and cooled starch-based foods (Raigond et al., 2014, Ahmad et al., 2016). RS3 is thermally stable and has a greater water holding capacity as compared to granular starch (Raigond et al., 2014). Studies have shown that autoclaving-retrogradation cycles together with α-amylase hydrolysis and degradation were more effective with the formation of RS3 (Lia et al., 2018).
- RS4 is known as the chemically modified starch by chemical treatments such as cross-linking, etherisation and esterification to resist hydrolysis and digestion by stomach acid and enzymes present in the digestive system (Bello-Pereza et al., 2018, Tacer-Caba and Nilufer-Erdilb, 2018). The main types of modification are conversions, substitutions as well as cross-linking. The chemical modification changes the structure and natural composition of different types of starch granules, therefore, increasing the resistance (Raigond et al., 2014).
- RS5 the fairly new category of resistant starch is an amylose-lipid complexes starch which is more susceptible to retrogradation, is described as resistant maltodextrins and are present in crops or food products that contain both starch and lipids where digestion occurs at a slow rate (Bello-Pereza et al., 2018).

2.4.3. Factors affecting the resistance of starch

The physical form of the legume, the structural morphology of the starch granules, amylose to amylopectin ratios and the interaction of starch with other food components such as ions, proteins, and lipids are examples factors affecting starch resistance (Zaman and Sarbini, 2015). Starch crystal characteristics, retrogradation of amylose and enzyme inhibitors also play a part in the resistance of starch. Granular morphology depends on the origin of the legume as each legume may have a different starch granule morphology. Smaller granules of starch are more susceptible to hydrolysis whereas larger granules due to a larger surface area are less susceptible to hydrolysis by enzymes to an extent. Shapes ranging from round to polyhedral also plays an important role as it affects the enzyme binding rate. The surface properties of the starch granule influence the resistance when the surface of the starch includes cracks, holes or crevices (Zaman and Sarbini, 2015).

The starch that contains a high amylose content is known to have a lower starch digestibility due to its compact linear structure. Retrogradation of amylose is one of the main factors for the formation of resistant starch and aids in resistance to enzymatic digestion (Ashwar et al., 2016). The association of starch with other food elements such as its relationship with proteins and lipids is another significant factor that affects the resistance. For example protein in rice interacts with starch and bind to the amylose and amylopectin causing a positive relationship amongst the protein and resistant starch (Yang *et al*., 2017). When starch encounters enzyme inhibitors such as polyphenols and lecithin, *in vitro* starch digestion is inhibited. An increase in enzymatic inhibitors decreases starch digestibility together with ions such as calcium, phosphates, and potassium which have been shown to prevent hydrogen bond formation amongst amylose and amylopectin chains, therefore, reducing the resistant starch content. Interaction between starch and lipids form complex amylose-lipid complexes that increase the resistant properties of the starch by reducing access of α- amylase to the amylose chain. The amylose-lipid compounds entangle the amylopectin thus restricting enzyme hydrolysis (Birt et al., 2013, Zaman and Sarbini, 2015).

2.4.4. Understanding the mechanism of the prebiotic effects of resistant starch

Greater awareness of the prebiotic function of RS may promote current efforts to improve public health by increasing their RS intake. RS is considered as an emerging prebiotic. Prebiotics are non- digestible food ingredients that stimulate the growth of beneficial bacteria in the gut (Yao et al., 2020). Prebiotics have been implicated in the improvement of the digestive system, improvement of cholesterol metabolism and reducing the risks of ulcerative colitis and colon cancer (Tacer-Caba and Nilufer-Erdilb, 2018). Prebiotics and resistant starch also occur naturally in many fruit and vegetables such as potatoes, bananas, legumes, and wheat (Zhang et al., 2015). For resistant starch to be classified as a prebiotic, it needs to fulfil the following criteria (Zaman and Sarbini, 2015):

- Resist enzymatic hydrolysis and acidic gastrointestinal conditions
- Be fermented by the good microbiota in the colon
- Stimulate the growth of good microbiota

The criteria outlined above are significant in the influence of resistant starch on the colon, as well as their prebiotic potential (Lockyer and Nugent, 2017, Yang et al., 2017).

Resistant starch is known to increases the growth of *Bifidobacteria* and *Lactobacillus* since resistant starch passes the entire small intestine without being hydrolysed by gastrointestinal enzymes and produces short-chained fatty acids and gases (Ashwar et al., 2016, Gibson et al., 2017). In a study done by Lia et al. (2018), the prebiotic effects of resistant starch from purple yam were evaluated on the proliferation of *Bifidobacterium adolescentis in vitro*. The results obtained showed that resistant starch stimulated the growth of the probiotic bacteria. According to Sarao and Arora (2017), useful prebiotics will follow the following criteria:

- Required in low dosages
- Low in calories
- Able be to easily be incorporated with food without drastically changed the properties of the food product
- Act as a good preservative
- Must be able to target the large intestine

Due to the characteristics of prebiotics i.e. non-digestible and stimulant of probiotic bacteria, there is a recent trend and development of using prebiotics as an encapsulating material. Some of the most common prebiotics are oligosaccharides, inulin, fructooligosaccharides, isomalto-oligosaccharides and recently resistant starch has been considered to potentially be a prebiotic (Sarao and Arora, 2017). In a study done by Liao et al. (2018), oligosaccharides, a prebiotic, was used as a co-encapsulating agent of *Lactobacillus fermentum* under gastrointestinal conditions as well as low temperatures. The results obtained showed that the prebiotic protected the cells in harsh environmental conditions, therefore displaying the potential to maintain probiotic survival without adversely affecting their release. Valero-Cases and Frutos (2015) conducted a study on the use of different concentrations i.e., 0, 1 and 2% of inulin as a reinforcement with sodium alginate and xanthan gum for microencapsulation. The results obtained showed that there was an improvement of the survival of *L. plantarum*, with 2% of inulin as compared to the other concentrations. According to a study by Yao et al. (2020), resistant starch can be used as an encapsulating membrane to enhance the survival of probiotics during storage and transition in the gastrointestinal gut. Using resistant starch as an encapsulating material for probiotic bacteria has answered and improved certain technological problems such as controlled release, heat and enzymatic stability and increased shelf life of sensitive biological compounds (Liu et al., 2020).

Muhammad et al. (2018) used resistant starch isolated from potatoes to encapsulate *Lactobacillus plantarum*, results obtained showed that the microencapsulation was efficient in easing the effects of lead toxicity in mice as compared to non-encapsulated. A study done by Zanjani et al. (2018) encapsulated *Lactobacillus casei* and *Bifidobacterium adolescentis* in various wall material such as wheat, calcium alginate, rice, and high amylose corn resistant starches and coated in poly L-lysine and chitosan. The microcapsules were incorporated into an ice cream and evaluated for viability over 100 days with results obtained suggesting that microencapsulation using high amylose corn resistant starch increased the survival of probiotics. Hi-maize starch is considered as a resistant starch, in a study by de Araújo Etchepare et al. (2016a), 1% resistant starch (Hi Maize) and 0.4% chitosan was added to alginate for microencapsulation of *Lactobacillus acidophilus* using the extrusion technique. The microparticles were subjected to simulated gastric juice and intestinal juice, with results showing resistant starch and chitosan to increase protection of probiotics in harsh environmental conditions.

The studies with the use of resistant starch as a co- encapsulating material as well as on its own, are increasing and can be used in several food and beverage applications. Different polysaccharides such as Hi-maize RS, maltodextrin considered as RS3 and gum arabic, was used in another study by Reyes et al. (2018), to determine their effects in the encapsulation of *Lactobacillus acidophilus* using spray drying and storage at different conditions i.e. 4°C and room temperature were explored. Microcapsules stored at 4°C were more viable that the ones stored at room temperature. Results have shown that the polysaccharides were effective in protecting the probiotic bacteria. Hi-maize resistant starch was used as an addition to sodium alginate as a wall material in a study done by Poletto et al. (2019b). The results obtained showed that the use of the hi-maize resistant starch improved the effectiveness of the microcapsules. The use of RS as wall material or reinforcement of the wall material is trending. Resistant starch increases protection and is known to benefit the host.

2.4.5. Fermentation of resistant starch and microcapsules

Gut microbiota present within the colon relies on energy produced through fermentation of the carbohydrates, which resist absorption and hydrolysis, in the upper gastrointestinal tract. Sources include soluble starch, non-starch polysaccharides such as celluloses, hemicelluloses, non-digestible oligosaccharides, and sugar alcohols.

The main fermentation pathway produces pyruvate from hexose sugars such as glucose, mannose, galactose, etc. that are present in resistant starch. When fermented the gut microbiota produces a range of short-chained fatty acids and gases. Resistant starch stimulates fermentation, therefore, increasing the bacterial count present in the colon which subsequently increased faecal mass and effects stool bulking (Brownawell *et al.*, 2012).

Resistant starch fermentation generally favours the production of butyrate. High levels of butyrate are found in people that are at low-risk to diet-related bowel diseases. This displays one of the many possibilities of resistant starch benefiting the health of consumers by stimulating the growth of bacteria and producing short-chained fatty acids(SCFA) (Bird et al., 2010, Lockyer and Nugent, 2017). Short-chained fatty acids (SCFA) are organic fatty acids which are formed within the large intestine due to the fermentation of polysaccharides, oligosaccharides, protein, peptides etc. SCFA and gas such as Carbon dioxide, methane and hydrogen are the main end products of fermentation. Studies have shown that the fermentation of starch yields acetate and butyrate, whereas the fermentation of pectin or gums yields in the production of acetate alone. This tells us that the fermentation that occurs within the colon is based and dependant on the composition of the substrate (Wong et al., 2006).

Different substrates support the growth of different types of bacteria and this, in turn, produces a different amount of Short-chained fatty acids. Resistant starch has the potential to increase the SCFA production which then improves the gut health (Nugent, 2005). At the end of the exponential growth, the phase is where the maximum production of SCFA occurs. The different types of SCFA that are produced are acetate, butyrate, propionate are abundantly found (Takagi *et al.*, 2016). Common SCFA can reduce the risk that digestive diseases, cancer and cardiovascular disease (Wong *et al.*, 2006). Acetate is one of the key SCFA in the blood and is used to monitor colon health. It is a primary substrate for cholesterol synthesis. Propionate is known to have a systematic effect on humans, especially in hypolipidemic action.

Butyrate is the major energy source for colonocytes and promotes a normal phenotype in these cells, potentially lowering the risk of diseases such as colorectal cancer as it regulates cell proliferation and differentiation (Vulevic et al., 2004, Wong et al., 2006). SCFAs serve as energy sources for the heart, brain, and body, improving bile salt solubility, mineral absorption, leptin development and control, all of which help protect against obesity and metabolism. (Ashwar *et al.*, 2016).

SCFA also regulates and improves blood flow in the colon, helps prevent the production of abnormal colonic cells and lowers the pH (Nugent, 2005, DeMartino and Cockburn, 2020). The use of RS influences the increase production of SCFA which has a wide range of benefits on the host.

2.5. Legumes

Legumes are known to be the' poor man's meat' in several places because of their protein content. The amino acids found in the protein can be used as development and repair support for many protein-based tissues in the body such as the heart, muscles, etc. In addition to providing food, legumes are also a source of vitamins and minerals (The National Research Council, 2006). Legumes are capable of enhancing the bioavailability of other nutrients as well as performing bioactive roles and serving as precursors to bioactive peptides, e.g. casein-peptides, which demonstrate different functions such as anti-thrombotic and anti-hypertensive behaviour (Duranti, 2006). Due to their low glycaemic index, they may be beneficial for preventing insulin-resistant diseases (Guillon and Champ, 2002).

According to Sandhu and Lim (2008), legume starches have been reported to have a higher amylose content and a lower digestibility as compared to cereal starches with starch fractions varying significantly throughout their digestibility. Moisture content present in legume starches varies between 3.12-16.00 %, whereas the lipid content between 0.04-1.40%, ash is between 0.03-0.65%, and nitrogen between 0.00-0.43% have been reported (Wani et al., 2016).

Toxic factors such as protease inhibitors and growth inhibitors that exist in certain legumes, but they are removed by different processing methods such as autoclaving, cooking and frying (Kasote et al., 2014). Processing of legumes have many positive effects i.e. it improves the nutritional value by inactivating toxic factors, increases the bioavailability of nutrients, improves the palatability of food and enhances sensory qualities (Tharanathan and Mahadevamma, 2003). Legumes are dehulled before processing, reducing cooking time, improving product characteristics and acceptability as well as the removal of tannins that affect protein digestibility.

2.5.1. *Vigna unguiculata*

Vigna unguiculata generally is known as cowpea belongs to the Leguminosae family. Cowpea a grain legume is native to Africa and is the most widely planted legume in Sub-Saharan Africa (Xiong et al., 2016). Cowpea may be used as animal feed and may also act as a cover crop that helps to reduce soil erosion (Sheahan, 2012). Cowpea may be able to improve the nutritional profile for many of those who are experiencing malnutrition, it is however regarded as a lost crop of Africa due to it not being intensively used to achieve its full potential in combatting malnutrition (Mtolo et al., 2017). Cowpea has a high nutritional profile and is rich in starch, protein, fat, and vitamins and minerals. Cowpeas are a valuable source of minerals such as potassium, phosphorus, magnesium as and selenium, and produce small amounts of zinc, copper, iron, sodium and manganese (Oyeyinka et al., 2020). Because of its high protein content and low-fat content, cowpea is a staple in many African countries and is eaten in many ways including as a whole in stews or the legume is milled into a meal and can be used in bakery items (Phillips et al., 2003, Oboh and Agu, 2009).

Cowpeas like other legumes are mostly composed of starch and protein. According to many previous studies the chemical composition of most cowpea exhibit the following approximate properties moisture (8-10%), ash (3-4%), protein (20-30%), fat (1-3%) and carbohydrates (40-60%)(Huang et al., 2007, Adebooye and Singh, 2008, Ashogbon and Akintayo, 2013, Ratnaningsih et al., 2016, Oyeyinka et al., 2020). Cowpeas are cooked and consumed in various ways across the sub-Saharan African region and may be used whole or as part of a meal e.g. soup or stew, it is the main ingredient in cowpea fritters and is also eaten with fermented cassava commonly known as "gari". Cowpea may also be milled into flour for the manufacture of baked goods. Cowpea may also be used as protein concentrates or isolates (Asare et al., 2013). Diabetic patients often consume legumes due to their low glycaemic index levels, with cowpea legumes shown to have a low glycaemic index (Oboh and Agu, 2009). Glycaemic index refers to the measurement of the impact of digested food that links to the level of blood glucose. The glycaemic load is the product of the glycaemic index and the number of carbohydrates consumed in a certain food.

A low glycaemic index may be due to variations in the structure of the starch and its amylose content. Hydrolysis index correlates with glycaemic index. Low glycaemic index is beneficial to the health as it reduces the risk of diabetes and may help control cardiovascular diseases (Oboh and Agu, 2009, Fitzgerald et al., 2011).

Cowpea starch may be used for a variety of food applications such as thickening or gelling agents and modifiers in food. Due to their high amylose content, they are very useful. With their ability to rapidly retrograde and resist hydrolysis during digestion however starch granule morphology may affect the physicochemical properties (Ratnaningsih *et al*., 2016). In a study done by Preet and Punia (2000) *in vitro* starch hydrolysis was carried out on four different cultivars of cowpea. The resistant starch obtained for each cultivar ranged 10-14% of the total starch present (g/100 g) of cowpea on dry matter basis. In another study done by Kasote *et al*. (2014) *in vitro* starch hydrolysis carried out on black and green gram yielded ±11% of resistant starch. Therefore, this study involves the isolation and characterization of resistant starch from cowpea for use as a wall material in microencapsulation of probiotics for food application.

Chapter 3: Physicochemical and structural characterization of resistant starch isolated from *Vigna unguiculata*

Abstract

In this study characterization was conducted on resistant starch, isolated from different cultivars of *V. unguiculata* (*Bechuana White, Fahari, PAN 311, TVU 11424 and DT129-4*). The fraction of resistant starch obtained was found in the range of 9.42-13.74%, with *DT129-4* yielding the highest fraction. Amylose content of the starches varied between 18.72-19.67%. Swelling power, solubility index and syneresis was directly proportional to the different temperatures. A correlation between amylose content and swelling power was observed as swelling power was indirectly proportional to amylose content. Water and oil absorption capacity was 31.93-88.06% and 10.29-27.71% respectively. Foaming, emulsion capacity, and stability were done, however, due to the lack of protein in the resistant starch samples, results obtained were relatively low, ranging between 0.00-22.41%. The degree of whiteness during colour analysis ranged between 80.81-84.61%. The FTIR-spectra displayed no distinctive difference amongst the vibration bands on the hydroxyl, methine and carbonyl frequency stretches and confirmed the polysaccharide nature of the resistant starch. The XRD spectra displayed a Ca-type crystalline structure for all 5 cultivars. Overall, this study shows that resistant starch has the potential to be used in many food applications and as a microencapsulation membrane.

3.1. Introduction

Legumes belong to the Leguminosae group of approximately 16000-19 000 known species and are considered to have beneficial physiological effects that help regulate and avoid different metabolic and or chronic diseases (Wani et al., 2016). A grain legume native to Africa, *Vigna unguiculata*, commonly known as cowpea, is the most widely planted legume in Sub-Saharan Africa. Most areas of Africa are subjected to poverty which is a leading cause of malnutrition (Mtolo et al., 2017). Cowpea is a protein, fat, carbohydrate, mineral and vitamin source (Ratnaningsih et al., 2016). Cowpea may be able to improve the nutritional profile for many malnourished people; however, Cowpea is considered a lost crop in Africa because it is not intensively used in the fight against malnutrition (Naiker et al., 2019). Cowpea is an important legume that is a good, readily available and cheap source of protein (16-31%) and carbohydrate (50-66%) (Oyeyinka et al., 2020). Cowpeas are also good sources of fibre, iron, zinc and contain substantial amounts of bioactive compounds.

Cowpea is a rich source of starch and has many potential applications within the food sector (Oyeyinka et al., 2020). Cowpea starch may be useful to manufacture gelling agents, thickeners as well as textural modifiers and may be incorporated in food application due to their rapid retrogradation and resistance to acid and enzyme hydrolysis (Ratnaningsih et al., 2016).

Starch occurs naturally and is biodegradable, cheap and the most abundant polymeric biomolecules that are present in legumes. It consists of 2 types of molecules i.e. amylose and amylopectin (Yu et al., 2014). Amylose and amylopectin differ greatly in structure, as well as properties and functionality, with amylose having a compact linear structure whereas amylopectin is branched. The amylose content of legume starches ranges from average to high, from 17.00 to 51.69% (Wani et al., 2016, Hoover et al., 2010). Resistant starch is considered as the total amount of starch that resists enzymatic and acidic hydrolysis and absorption within the upper gastrointestinal tract, therefore, accumulating within the large intestine (Topping et al., 2003, Jyothsna and Hymavathi, 2017). For the natural beneficial microbiota present in the colon, resistant starch acts as a prebiotic which stimulates growth (Gibson et al., 2017). The starch undergoes fermentation by the microbiota and short-chained fatty acids are produced. Resistant starch does not increase blood sugar and has comparable physiological effects to dietary fiber (Yang et al., 2017). Resistant starch has been reported to reduce the risks of cardiovascular disease, circulating growth factors, reduce inflammation and reduces oxidative stress (Jyothsna and Hymavathi, 2017).

There are many factors such as physical and functional properties to be taken into consideration before the utilization of an ingredient in food applications. Physical characteristics include morphological characteristics such as size and particle distribution, scanning electron microscopy, Fourier transform infrared (FT-IR) spectroscopy as well as X-Ray diffraction. Functional properties include amylose content, swelling power and solubility, syneresis, water and oil absorption capacities, foaming properties, emulsifying properties and other components. Factors like environmental factors, harvest methods, post-harvest storage and genotype, affect the characteristics of starch and may sometimes have significant influence over the genotypic difference (Mir et al., 2017). Due to high demand for new functional ingredients within the food sector and an increased interest in resistant starch, this study provides a physical and functional characterization of resistant starch isolated from 5 different cultivars of cowpea obtained through *in vitro* simulated gastrointestinal digestion for potential food applications.

3.2. Materials and methods

3.2.1. Starch isolation

The five different cultivars (*Bechuana White*, *Fahari*, *PAN 311*, *TVU 11424*, *DT 129-4*) of cowpea were soaked, at a ratio of 1:3 (m/v), in distilled water (pH 7) and left overnight. The soaked cowpea was then dehulled and left in an incubator at 37°C to dry overnight. Once the seeds were dry, they were milled, using a Kenwood grinder, and sifted using a 180 μm sieve and then transferred to a zip-lock bag for storage prior to analysis.

The flour was dispersed into hexane at a ratio of 1:3 (m/v) and then left to defat for 3 h at 250 rpm, thereafter the solution was centrifuged at 5000 x g for 15 min and the hexane supernatant decanted. The pellet was steeped into 0.025 M Na_2SO_3 at a ratio of 1:10 (w/v). The mixture was homogenized, and the suspension placed at room temperature for 1 h. The samples were then centrifuged at 5000 x g for 15 min and washed with ethanol twice. The ethanol slurry was centrifuged at 5000 g for 15 min and then be washed with distilled water and centrifuged at 5000 g for 15 min. The pellet was left to dry overnight in the laminar stored in a zip-lock bag prior to analyses (Guo et al., 2019).

3.2.2. Isolation of resistant starch under simulated *in vitro* gastrointestinal digestion

According to Mahasukhonthachat (2010) and Phongthai et al. (2018) 10 mL of artificial saliva from porcine α-amylase (Sigma Aldrich - A3176) was used to treat 10 g of starch for 10-15 s, thereafter 50 mL of pepsin (Sigma Aldrich - P7000) was added to solution and incubated at 37°C for 1 h in a reciprocating water bath at 85 rpm. 250 mL of 0.2 M sodium acetate was added to neutralise the solution and adjust the pH to 6. A 50 mL mixture of pancreatin (Sigma Aldrich-P7545) and amyloglucosidase (Sigma Aldrich-A7255) was added, and the solution was incubated 37°C for 120 min in a reciprocating water bath at 85 rpm. Starch hydrolysis (%) was determined by quantification of glucose in the digesta between 0 and 120 min of digestion using a Glucose Oxidase-Peroxidase kit (Sigma Aldrich – GAGO20). The factor conversion from glucose to starch content used was 0.90. Following incubation, the solution was centrifuged at 8000 x g for 20 min, the supernatant was discarded, and the pellet freeze dried and then transferred to zip lock bags for further analysis.

3.2.3. Characterization of starch

3.2.3.1. Particle size distribution

2 mg of isolated resistant starch sample was dispersed in distilled water using 1.59 as refractive index of the standard material polystyrene latex similar to Coghetto et al. (2016). The particle size distribution of the suspension was analysed using a laser light scattering particle analyser at 658 nm (Litesizer 500, Anton Paar., Graz, Austria).

3.2.3.2. Scanning election microscope (SEM)

Resistant starch samples were mounted on stubs, coated with gold with a thickness of 10-20 nm using a Quorum K150 RES Sputter Coater and were viewed with a LEO 1450 SEM at an accelerating voltage of 5 kV.

3.2.3.3. X-Ray Diffraction

X-ray diffraction analysis was conducted using an X-ray diffractometer (D8 Advance, BRUKER AXS, Germany) measuring continuous ϑ-ϑ scan in locked coupled mode. With a tube voltage of 40 kV, tube current of 40 mA, variable slits of V20 variable slit at 2ϑ range available: 0.5° to 130° with a step size of 0.034°.

3.2.3.4. Fourier transform infrared (FTIR) spectroscopy

Spectroscopic Analysis of resistant starch samples was done by Fourier Transform Infra-Red Attenuated Total Reflectance (ATR-FTIR). All 5 samples of cowpea cultivars were scanned from 300 to 4 000 cm^{-1} on a Perkin Elmer Series 100 spectrometer equipped with a universal sample diamond accessory. Each ATR-FTIR spectrum sample scan was preceded by a background scan using air as a blank. All samples were scanned at 23±1°C

3.2.4. Physicochemical characteristics

3.2.4.1. Amylose content

The amylose content for cowpea samples were determined using a method by Hamid et al. (2015) with minor modifications. Briefly, 10 mL of 0.5 M KOH was added to 100 mg of flour samples, the solution was transferred into a 100 mL volumetric flask made up to the mark with distilled water. 10 mL of that solution was transferred into a 50 mL volumetric flask where 5 mL of 1.0 M HCL followed by 0.5 mL of 20% iodine reagent added.

The volume was made up to the mark with distilled water to 50 mL and allowed to stand for 5 min. The absorbance was then measured at 625 nm and the amylose content determined from a standard curve developed using amylose and amylopectin blends from potato starch

3.2.4.2. Swelling and Solubility Index

According to Guo et al. (2019) samples were incubated for 1 h in a water bath at 70, 80, and 90°C after which samples were centrifuged at 5000 x g for 10 min. Swelling index is expressed as the gain in mass of tubes (g/g). The solubility index (g/g). was expressed as the gain in weight of the supernatants that were dried in pre-weighed petri dishes at 110°C for 12 h.

3.2.4.3. Syneresis

Using a method described by Guo et al. (2019) suspensions were heated at 90°C for 30 min and were then incubated at 4°C. Syneresis was determined over a 120 h period and calculated as the percentage of water released after centrifugation at 3000 x g for 15 min.

3.2.4.4. Water/Oil Absorption Capacities

As per Naiker et al. (2019), flour sample were massed in centrifuge tubes. Distilled water and refined oil were added to separate centrifuge tubes and vortexed for 5 min. The slurries formed, were centrifuged at 3000 x g for 10 min. The water or oil released following centrifugation was massed.

3.2.4.5. Colorimetric analysis

Colour analyses was conducted using a colorimeter (ColorFlex EZ, Hunter Lab- SCFEZ 0840). The instrument was standardized with Hunter lab colour standards, and Lightness (*L**), redness to greenness (a*) and yellowness to blueness (*b**) values were measured. *Whiteness* was calculated according to Oyeyinka et al. (2015).

3.2.4.6. Foam Capacity and Stability

As per methods by Naiker et al. (2019) with minor modifications 2% sample solution (m/v) was subjected to shearing for 3 min to activate foam structure. Contents were then transferred to a measuring cylinder, after 30 s foam volume was calculated using the following equations:

$$\text{Foam Capacity (\%)} = \frac{\text{Vol. after shear (mL)} - \text{Vol. before shear (mL)}}{\text{Vol. before shear]}}$$

Foam stability (%) was calculated after allowing contents in measuring cylinder to stand for 60 min at room temperature

$$\text{Foam Stability (\%)} = \frac{\text{Vol. of foam (mL)at 60 min}}{\text{Initial foam (mL) vol.}} \times 100$$

3.2.4.7. Emulsifying Properties

Emulsifying properties of flour sample was determined using a method by Naiker et al. (2019). Refined oil was added to the flour samples and the solution homogenised.

The solution was centrifuged at 1500 x g for 5 min and the height of the emulsified layer and the total height recorded. The emulsion was then heated at 80°C for 30 min, centrifuged at 1500 x g for 5 min and calculated using the following equations:

$$\text{Emulsion Capacity (\%)} = \frac{\text{Height of emulsified layer (mL)}}{\text{Total height of tube contents (mL)}} \times 100$$

$$\text{Emulsion Stability (\%)} = \frac{\text{Height of emulsified layer after heat (mL)}}{\text{Height of emulsified layer before heating (mL)}} \times 100$$

3.2.5. Statistical analysis

Statistical analysis was done using GraphPad Prism (5). Results were represented as the mean ± standard deviation where n =3. Tukey's Multiple Comparison test and confidence level: 0.05 (95% confidence interval) was used to compare the means by one-way analysis (ANOVA).

3.3. Results and discussion

Starch can be classified into different fractions which are total starch (TS), rapidly digested starch (RDS), slowly digested starch (SDS) and resistant starch (RS). Within 20 min RDS can be hydrolysed to glucose while SDS is hydrolysed to glucose in 120 min when subjected to treatment with digestive enzymes during in an *in vitro* assay, the starch that remains after 120 resists hydrolysis and is therefore known as the resistant starch (Englyst et al., 1992, Englyst et al., 1995). The percentage of total starch hydrolysed at given times i.e., 20 and 120 min are calculated as the starch fractions. According to Table 3.1 the total starch per cultivar varied and were found in the range 57.60-65.67% with cultivar *Fahari* having the highest total starch value. The starch fraction per cultivar varied for RDS, SDS and RS which ranged between 39.15-53.15%, 1.27-10.49%, 9.42-13.74%.

According to Raigond et al. (2014) rapidly digested starch and slowly digested starch and resistant starch are generally inversely proportional to each other, this can be observed in the results obtained especially with cultivar *Bechuana White* and *Pan 311*. The highest percentage of resistant starch was observed in *DT129-4* (13.74%) with *Pan 311* having the lowest percentage (9.42%). In a study done by Kasote et al. (2014) similar findings were observed for resistant starch content from pigeon pea, green gram and black gram which was found to be 16.86%, 11.60% and 11.35% respectively.

In another study by Sasanam et al. (2011) the resistant starch content for decorticated black cowpea, white cowpea, red cowpea and red kidney beans were 4.59%, 4.63%, 10.36% and 8.97% respectively. Resistant starch poses many potential health benefits and may be regarded as a prebiotic. Variation in resistant starch yield as can been seen between *PAN 311* and *DT 129-4* may be due to structural factors such as starch granule morphology, such as granule shape, size and surface are some of the characteristics that influence the digestibility of starch. Granules which are smaller in size and contain crevices which serves as entry points for enzymes and acid are more susceptible to hydrolysis. Interaction of starch with proteins, lipids, carbohydrates as well as temperature and genetic makeup are also factors that affect digestibility (Raigond et al., 2014, Zaman and Sarbini, 2015).

Table 3.1: Physicochemical properties of resistant starch isolated from cowpea cultivars

Starch fractions (%)	*Bechuana White*	*Fahari*	*PAN 311*	*TVU 11424*	*DT129-4*
Total starch	64.76±1.12^{a}	65.67±1.18^{a}	59.84±0.69^{b}	57.60±0.61^{c}	65.04±0.68^{a}
Rapidly digested	53.15±0.54^{a}	47.93±0.57^{b}	39.15±0.41^{d}	41.08±5.92^{d}	45.48±0.31^{c}
Slowly digested	1.27±0.42 ^{a}	5.54±1.17^{b}	10.49±0.57^{c}	2.02±059^{a}	5.03±1.69^{b}
Resistant Starch	10.30±0.31^{a}	10.78±0.62^{a}	9.42±0.16^{a}	12.48±0.58^{b}	13.74±1.40^{c}

All data is expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$).

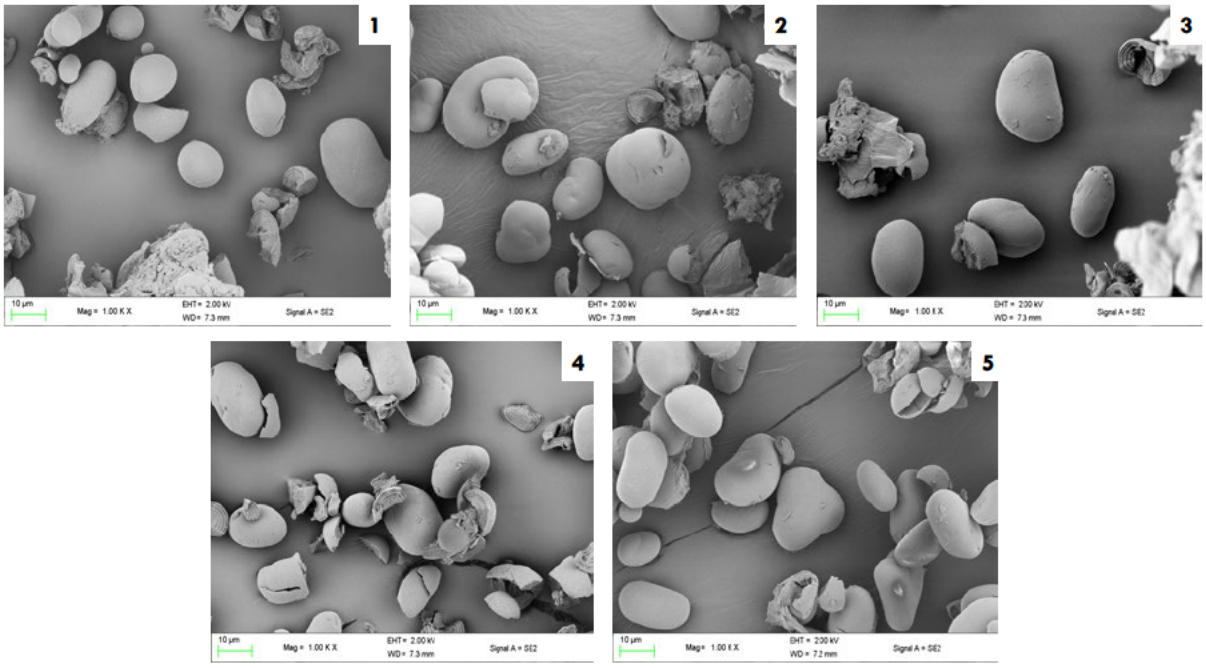

Figure 3.1: Scanning Electron Micrographs (SEM) of resistant starch isolated from *Vigna unguiculata* cultivars (1: *Bechuana White*, 2: *Fahari*, 3: *Pan 311*, 4: *TVU 11424* and 5: *DT129-4*) [magnification 1000x].

The starch granules for all cultivars displayed a similar granule shape, an oval to spherical shape while some were round but irregular in shape. The granules are approximately 10-20 μm in length. The surface of resistant starch granules for *Bechuana White*, *Fahari*, *Pan 311* and *DT129-4* were smooth without holes or crevices however cultivar *TVU 11424* displayed a few serrations and indentations on the surface of resistant starch granules which may have attributed to a high swelling power, as shown in Table 3.4. According to Bird et al. (2009) SEM has been used to determine the granule characteristics of resistant starch. The granule structure differs depending on the type of resistant starch as well as their processing treatments.

There are two main types of enzymatic attack during hydrolysis as mentioned by Bird et al. (2009) are exo-corrosion hydrolysis and endo-corrosion hydrolysis. Exo-corrosion occurs when enzymes alters the surface and erodes sections of the granule surface while endo-corrosion is associated with enzyme penetration of the granule and erosion of internal parts (Bird et al., 2009). From the results shown in Figure 3.1 exo-corrosion can be observed with broken starch granules in all 5 images which may be attributed to milling methods. Cultivar *DT129-4* displayed most favourable characteristics with cultivar *TVU 11424* the least. According to Guo et al. (2019) morphology of starch granules are in relation to physicochemical properties such as pasting properties, water absorption capacity as well as swelling and solubility, syneresis and amylose content.

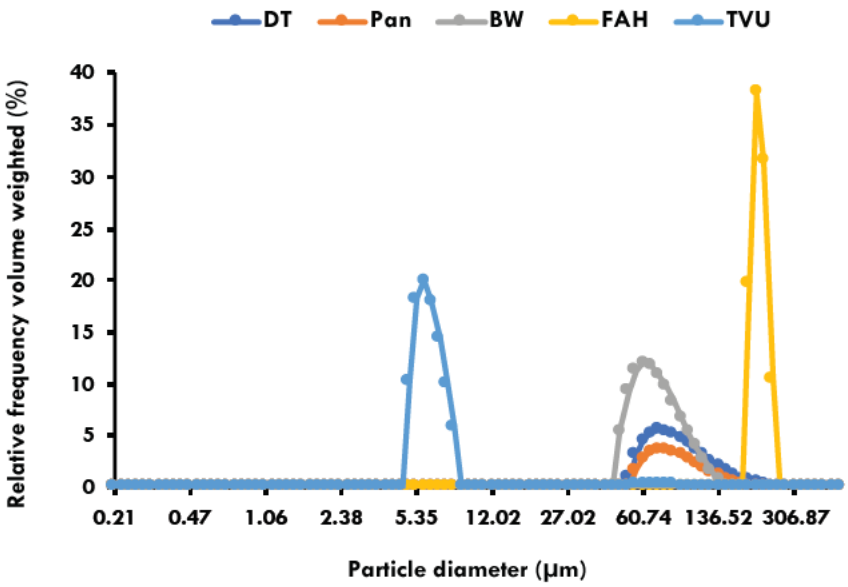

Figure 3.2: Particle Size Analysis of resistant starch isolated from *Vigna unguiculata* cultivars [DT: *DT129-4*, Pan: *Pan 311*, BW: *Bechuana White*, FAH: *Fahari* and TVU: *TVU 11424*].

Particle size analysis was conducted to compare the particle size of each cultivar. Fine samples display a smaller particle size. In the results obtained showed that the particles of all 5 cultivars varied vastly and ranged between 5-200 µm. This could be due to variation in milling and sieving. As shown in figure 3.2 cultivar *TVU 11424* had the lowest particle size of 5 µm while *Fahari* had the greatest particle size of 200 µm. According to Mahasukhonthachat (2010) finer samples contain mechanically separated starch granules while multicellular samples are coarse. The resistant starch isolated from cowpea cultivars displayed a similar morphology however the granules per cultivar varied in size and shape with each image.

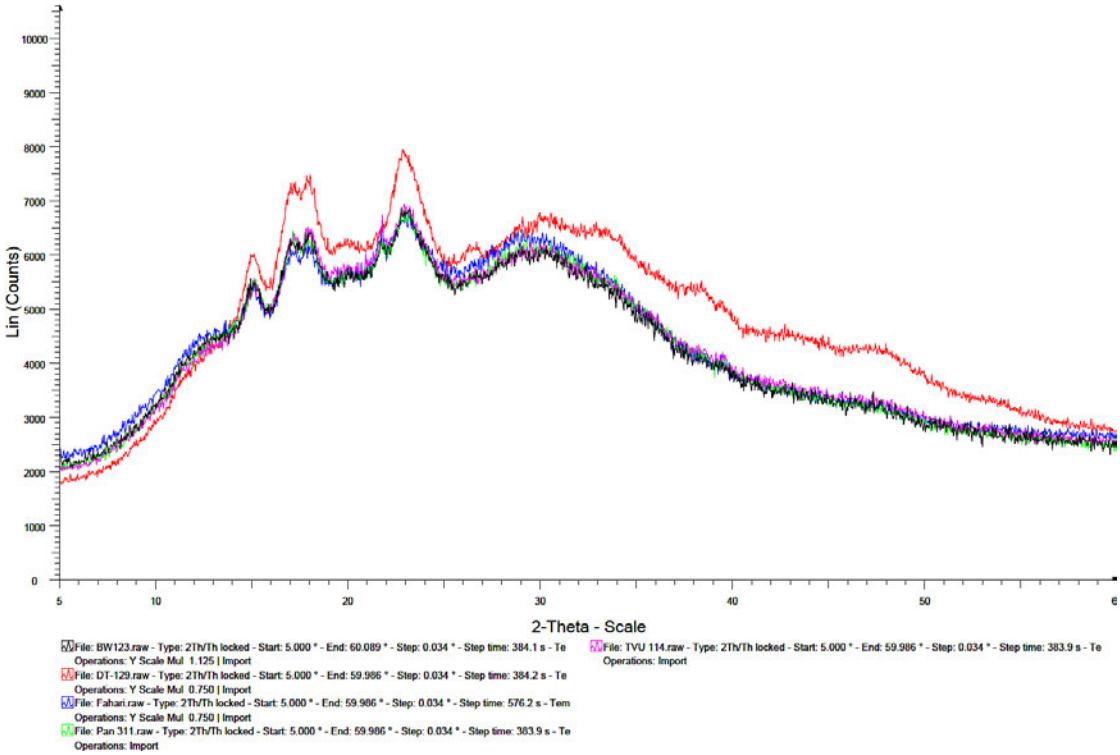

Figure 3.3: X-ray diffraction spectra of resistant starch samples isolated from *Vigna unguiculata* cultivars [A: *Bechuana White*, B: *Fahari*, C: *Pan 311*, D: *TVU 11424* and E: *DT129-4*].

X-ray diffraction was applied to determine the presence and the type of crystalline structure of resistant starch granules and the crystallinity of RS obtained from various botanical sources which may be formed under numerous conditions (Ma and Boye, 2017). In the diffraction spectra of the resistant starch of the five cultivars, there were five strong peaks at starting at 5° and moving on to 15, 17, 18 and 23° (2θ) (Figure 3.3).

All the results obtained were similar with the exception of *DT129-4*, which had a significantly higher peaks than the other four cultivars. The results obtained were similar to previous crystallinity studies done on pulses and legumes. Starch granule are considered to be semi-crystalline structures and fall into 3 categories of crystallinity i.e. A, B and C-types (Tester et al., 2004). A-type is usually found in cereals and grains while B-type is usually found in tubers and roots like potatoes and yam ad C-type in peas and legumes (Guo et al., 2019). According to the spectra these starches displayed a C-type crystallinity pattern which is interposed between type A and B with the pattern of C-type starch gives strong single peaks at approximately 17 and 23° (2θ), and a few small peaks at around 5.6 and 15° (2θ) which can be observed in the results obtained for the five cultivars of cowpea starch (Ratnaningsih et al., 2016).

Similar peaks were observed in a study done by Acevedo et al. (2019) which analysed the crystalline structure of pigeon pea (C*ajanus cajan*) and dolichos bean (*Dolichos lab-lab*). According to Ratnaningsih et al. (2016) in a study done by Hizukuri (1985) it was hypothesized that the C-type can be broken down into CA- type which is closer to A-type and CB-type which is closer to B-type. The CA-type has a shoulder peak around 18° (2θ) and a strong single peak at 23° (2θ) while the CB type has 2 shoulder peaks at 22 and 24° (2θ). From this study, the resistant starch of five cowpea cultivars can be grouped into the CA-type.

ATR-FTIR analysis of the samples was done to determine the stretching frequencies of 5 samples of cowpea cultivars to characterize the chemical bonds or chemical groups that are related to specific structural organization of starch granules of resistant starch isolated from cowpea cultivars. The absorption peaks at the O-H stretch for each cultivar were between 3278.26-3291.07 cm^{-1}, this indicated the complex vibration stretching contained free, inter- and intra- molecular O-H (hydroxyl) groups whereas the absorption peaks at the C-H stretching were between 2931.41-2933.45 cm^{-1} were contributed by the methine stretching. The absorption peak at the C-O and C-O stretch were between 1631.45-1643.73 cm^{-1} and 997.27-1005.35 cm^{-1} respectively. There was no distinctive difference amongst the vibration bands on the hydroxyl, methine and carbonyl frequency stretches, this may be attributed to similar chemical structures, therefore, resulting in similar absorption peaks however cultivar *DT129-4* can be seen with the best peaks as compared to the other cultivars. This shows that *DT129-4* has a stronger crystalline structure as compared to the other cultivars. In a study done by Guo et al. (2019) ATR-FTIR was done on chestnuts from different regions in china exhibited similar peak values to the values obtained in this study. Similar peaks were also obtained in a study conducted by Hussain et al. (2019) on ATR-FTIR of resistant starch isolated from chestnut flours.

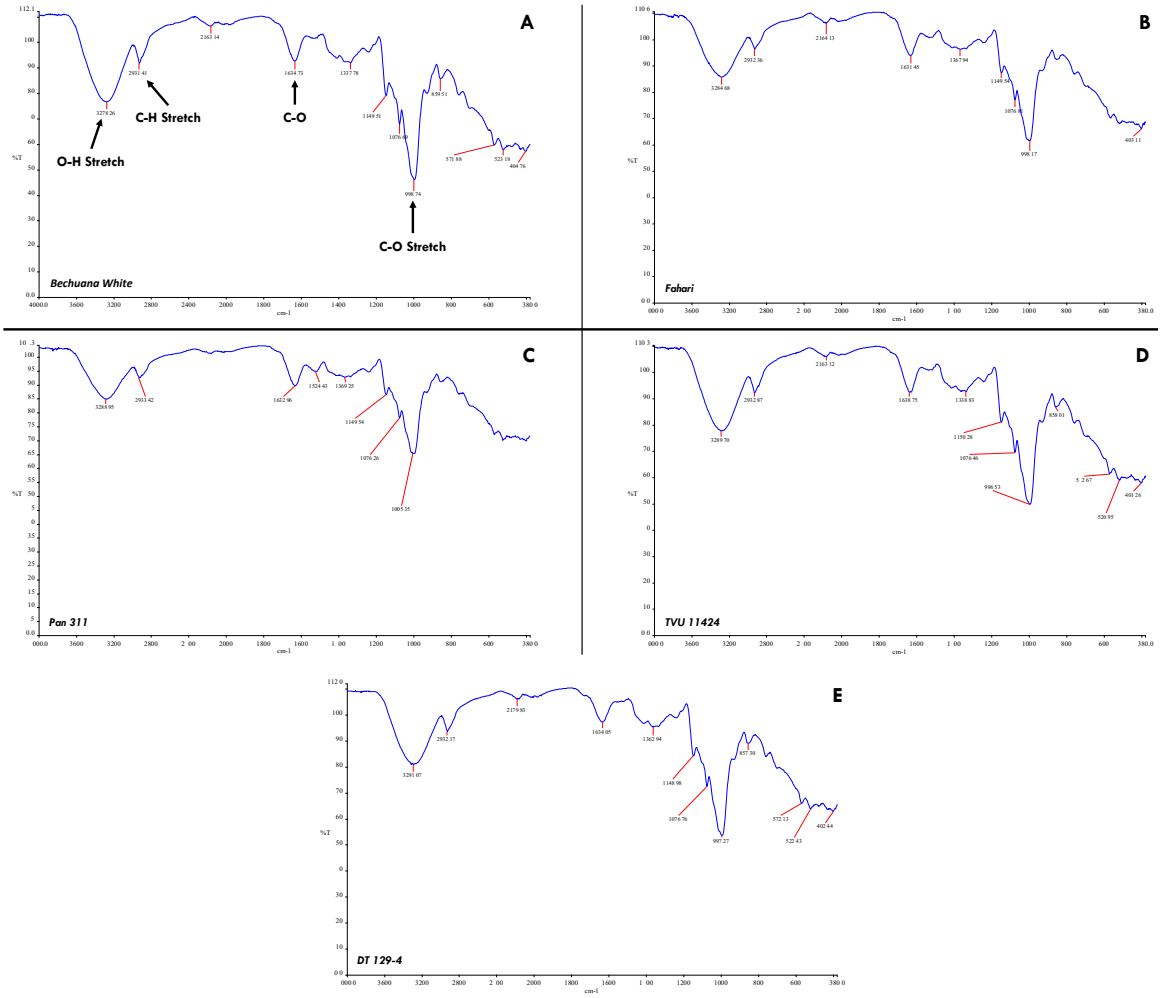

Figure 3.4: Fourier transform infrared (FT-IR) spectra of resistant starch samples isolated from *Vigna unguiculata* cultivars [A: *Bechuana White*, B: *Fahari*, C: *Pan 311*, D: *TVU 11424* and E: *DT129-4*].

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Physicochemical properties of resistant starch are extremely important for food application purposes, with a significant difference between resistant starch samples for cultivars tested. The amylose content of resistant starch samples were found between 18.72-19.90%, with cultivar *Bechuana White* having the lowest amylose content and *PAN 311* having the highest amylose content (Table 3.2), results are similar to findings for amylose content observed in a study by Naiker et al. (2019).

In a study by Sirivongpaisal (2008) the amylose content for Bambara groundnut was 21.67% similar to the results obtained for cowpea. In another study by Acevedo et al. (2019) showed that the amylose content for pigeon pea (*Cajanus cajan*) and dolichos bean (*Dolichos lab-lab*) were 26.24 and 21.18% respectively. According to Ashogbon and Akintayo (2013) the amylose content of white cowpea and brown cowpea seeds were reported to be 22.06 and 26.53% respectively. Functional properties like swelling power, crystallinity and gelatinisation are directly impacted by the amylose content (Oyeyinka et al., 2015).

Water and oil absorption capacity is the ability of starch to absorb water or oil and is an important component with regard to beverage applications (Table 3.2). The hydrophilic sites in starch chains, allows for interaction with water through hydrogen bonding. The interaction between amylose and amylopectin chains with water is considered weak at standard room temperature, but when the temperature rises, the thermal energy appears to weaken the hydrogen bonds and increase the engagement with water, resulting in the assimilation of starch and water (Wani et al., 2016). The water absorption for cultivars tested was found in the range of 31.93-88.06%. There was a great variation amongst the cultivars with *PAN 311* having the lowest water absorption capacity and *TVU 11424* having the greatest water absorption capacity.

Water binding capacity of starch is important in the baking industry as it determines if the flour can be used in formulations involving dough and is also an indication of the amount of water available for gelatinisation with a lower absorption capacity desirable for a thinner consistency (Alka et al., 2012). Oil capacity ranged from 10.29-23.81%. This may be due to these cultivars having a similar lipid or hydrophobic groups present, however, cultivar *Bechuana White* had the greatest oil absorption capacity as compared to the other cultivars. Both water and oil absorption capacities impact consistency, flavour and mouth feel (Naiker et al., 2019). The variation between water and oil absorption capacity could be attributed to environmental factors during growth and differences in harvest and storage as well as genetic make-up of each cultivar (Florence and Urooj, 2015).

Foaming capacity (Table 3.2) amongst all cultivars varied and was found in the range of 5.00-16.67%, with cultivar *Bechuana White* having the highest foaming capacity and stability while the other cultivars had no foaming stability. Foaming stability for each cultivar was found in the range of 0.00-13.89%. Foaming properties of starch correlate with the amount of protein and its solubility. According to Wani et al. (2015) three factors, i.e. the transport, penetration and reorganization of the molecules at the air-water interface, decide the formation of foam.

Emulsion capacity (Table 3.2) is the total amount of oil that undergoes emulsification by protein dispersion, while emulsion stability is the potential of the emulsion produced to remain unchanged (Wani et al., 2015). Emulsion capacity and stability were found in the range of 4.44-7.78% and 0.00-22.41% respectively. However, since only resistant starch was isolated from cowpea cultivars, the low values observed for foaming and emulsion capacity and stability could be attributed to the lack of protein present.

Table 3.2: Physicochemical properties of resistant starch isolated from cowpea cultivars

Physicochemical properties	*Bechuana White*	*Fahari*	*PAN 311*	*TVU 11424*	*DT129-4*
Amylose content (%)	18.72±0.95^{a}	19.67±0.63^{a}	19.90±0.57^{a}	18.93±0.53^{a}	19.53±0.20^{a}
WAC (%)	67.07±1.11^{a}	69.95±1.01^{a}	31.93±0.81^{b}	88.06±0.47^{c}	35.26±0.75^{b}
OAC (%)	23.81±0.86^{a}	17.77±0.40^{b}	27.71±0.33^{a}	11.39±0.37^{c}	10.29±0.21^{c}
Foaming capacity (%)	16.67±2.89^{a}	11.67 ± 2.89^{a}	8.33±5.77^{b}	6.67±2.89^{b}	5.00±0.00^{c}
Foaming stability (%)	13.89±12.73^{b}	0.00 ±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Emulsion capacity (%)	7.50±0.83^{a}	5.56±0.96^{b}	5.56±0.96^{b}	7.78± 0.96^{a}	4.44±4.19^{b}
Emulsion stability (%)	22.41±2.5^{a}	0.00±0.00^{b}	19..44±7.35^{a}	21.67±2.89^{a}	0.00±0.00^{b}

All data is expressed as mean ± standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$).

Colour analysis (Table 3.3) was done on all cultivars, where "*L*" determines the lightness, "*a*" determines the redness to greenness and "*b*" determines the yellowness to blueness. The lightness of each cultivar was in the range of 81.94-85.14. Similar results were obtained in a study done by Naiker et al. (2019) on cowpea samples. A higher value for "*L*" was observed for *Bechuana White* this indicated that this cultivar was whiter as compared to other samples. The value obtained for "*a*" and "*b*" were in the range of 0.04-0.88 and 1.36-6.45. Colour analysis is important for food application as a difference in colour may impact the final colour of food products. Colour variability may be due to the different types of colour components as well as particle size and multiple contact points (Naiker et al., 2019).

Table 3.3: Colour evaluation of resistant starch samples from cowpea cultivars

Colour properties	*Bechuana White*	*Fahari*	*PAN 311*	*TVU 11424*	*DT129-4*
L*	85.14±0.08^{a}	81.94±0.05^{a}	82.99±0.03^{a}	82.27±0.05^{a}	84.67±0.02^{a}
a*	0.50±0.01^{a}	0.63±0.03^{a}	0.70±0.01^{a}	0.88±0.03^{a}	0.04±0.01^{b}
b*	4.71±0.01^{a}	6.45±0.05^{a}	5.93±0.02^{a}	5.24±0.05^{a}	1.36±0.01^{b}
Whiteness	84.41±0.00^{a}	80.81±0.00^{b}	81.97±0.00^{b}	81.49±0.00^{b}	84.61±0.00^{a}

All data is expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$).

Swelling power and solubility index (Table 3.4) observed for all cultivars were directly proportional to the temperatures. The swelling indices (g/g) observed at 70, 80 and 90°C were 0.53-0.84, 1.29-1.70 and 1.7-2.21 respectively. And the solubility indices observed at 70, 80 and 90°C were 0.1-0.2, 0.2-0.5 and 0.6-0.7 respectively. Swelling power is related to the pasting characteristics of the resistant starch which includes gelatinisation and retrogradation whereas solubility index refers to the amount of starched soluble at various temperatures (Guo et al., 2019). Cultivar *TVU 11424* had the greatest swelling power and solubility index at 90°C with *Fahari* having the lowest. All cultivars varied for swelling power, however, there wasn't a significant difference observed for solubility index. Variation in swelling and solubility could be attributed to variation in starch granule morphology including particle size distribution, surface area properties as well as crystallinity, water absorption capacity and amylose content (Naiker et al., 2019). According to Hamid et al. (2015) starch with lower amylose content swell more as compared to starches with higher amylose content. Therefore, the correlation between amylose content and swelling can be observed with the results depicted in Table 3.2 with the amylose content and swelling power of cultivars *TVU 11424* and *Bechuana White*.

Table 3.4: Swelling power and solubility of resistant starch samples from cowpea cultivars

		Temperature (°C)		
		70	**80**	**90**
Swelling power (g/g)	*Bechuana White*	0.84±0.07^{b}	1.35±0.04^{a}	2.04±0.01^{a}
	Fahari	0.66±0.06^{a}	1.54±0.12^{a}	1.7±0.06^{b}
	PAN 311	0.54±0.06^{a}	1.29±0.06^{a}	1.93±0.09^{a}
	TVU 11424	0.77±0.08^{b}	1.7±0.12^{b}	2.21±0.07^{a}
	DT -129	0.53±0.06^{a}	1.54±0.03^{a}	1.97±0.04^{a}
Solubility (g/g)	*Bechuana White*	0.02±0.00^{a}	0.04±0.00 ^{a}	0.06±0.00 ^{a}
	Fahari	0.02±0.00^{a}	0.04±0.00^{a}	0.06±0.00^{a}
	PAN 311	0.02±0.00^{a}	0.02±0.00^{b}	0.06±0.01^{a}
	TVU 11424	0.02±0.00^{a}	0.03±0.00^{a}	0.07±0.00^{a}
	DT -129	0.01±0.00^{a}	0.05±0.01^{a}	0.06±0.00^{a}

All data is expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$).

Syneresis is the spontaneous release of liquid from a gel which is an index of the degree of retrogradation at low temperatures i.e. 4°C. Syneresis is considered an undesirable property of legume starches for food application. Degree of syneresis can be seen as directly proportional to the storage time in low temperatures. Over a 120 h, the degree of syneresis was in the range 12.08-20.00 %, with cultivar BW having the highest degree of syneresis (Table 3.5). The increase in syneresis may be attributed to an increase in intra- and intermolecular hydrogen bonding that occurs when chains aggregate when the temperature decreases, therefore, causing a spontaneous release of water from the gel (Hamid et al., 2015). Variation in the degree of syneresis may vary amongst cultivars due to variation in chemical composition as well as intermolecular interactions of amylose chain starch (Wani et al., 2016)

Table 3.5: Syneresis of resistant starch samples from cowpea cultivars

	Syneresis (%)				
Time (h)	***Bechuana White***	***Fahari***	***PAN 311***	***TVU 11424***	***DT129-4***
24	2.78±0.48^{a}	0.56±0.96^{b}	1.11±1.27^{a}	1.67±0.83^{a}	2.78±0.48^{a}
48	4.44±0.48^{a}	3.61±1.27^{a}	4.17±0.83^{a}	6.39±1.27^{a}	5.56±1.73^{a}
96	15.83±0.83^{b}	10.00±0.83^{a}	9.44±0.48^{a}	10.83±1.67^{a}	8.89±1.27^{a}
120	20.00±1.44 ^{b}	16.39±1.27^{b}	12.50±1.67^{a}	13.06±0.48^{a}	12.08±0.59^{a}

All data is expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$)

3.4. Conclusion

Physicochemical and structural properties are extremely important as it determines functionality in food application as well as interaction between other food components. *Vigna unguiculata* contains a considerable amount of resistant starch. The results obtained for physicochemical properties varied among all five cultivars, however similar results were obtained for structural properties, with cultivar *DT129-4* showing the most favourable results overall. Resistant starch is a growing interest in the food industry and is known to have a wide range of health benefits. From the results obtained one can confirm that resistant starch has a potential to be used in various food applications such as baked goods, snacks, and beverages. Recent academic interests include the use of resistant starch as a wall material for microencapsulation, which can be further investigated with regard to food applications.

Chapter 4: Microencapsulation of *Lactobacillus casei* and *Bifidobacterium animalis* using resistant starch from *Vigna unguiculata*

Abstract

In this study, the viability and stability of probiotics, *Lactobacillus casei* and *Bifidobacterium animalis*, encapsulated in a resistant starch medium through freeze-drying was evaluated together with particle size, SEM and colour analysis of microcapsules. The encapsulation yield after freeze-drying was between 81.55-88.78%, with viability of the microcapsules analysed in simulated gastric juice (SGJ) and simulated intestinal juice (SIJ) as well as in apple juice stored at 4°C over a 28 d period. The microencapsulated probiotics in this study had a substantial final count for SGJ and SIJ between 7.30-7.98 Log CFU/mL and 7.26-7.89 Log CFU/mL respectively. The Log CFU/mL for free cells were significantly lower than the microcapsules. The final viability for microcapsules in the juice at the end of 28 d for RSL, RSB and RSLB were 7.53, 6.98 and 7.46 Log CFU/mL. This confirms that microencapsulation enhanced the survival of *Lactobacillus casei* and *Bifidobacterium animalis* in fruit juice under cold storage. Results from this study show that resistant has potential as an encapsulating material as it protected the probiotic cells in conditions such as freeze-drying, simulated gastrointestinal digestion and low pH environments during beverage application.

4.1. Introduction

The use of probiotics and prebiotics have been trending in the food industry with an increase in functional foods such as probiotic based food products and beverages due to associated benefits (Afzaal et al., 2020). Probiotics are non-pathogenic, live microorganisms that are considered to be beneficial to the host body as they aid in digestion and reduces the symptoms of many chronic gastrointestinal diseases such as Chron's diseases, ulcerative colitis and irritable bowel syndrome (Muhardina et al., 2018). They maintain the microbial environment in the colon and as well as the intestinal barrier of the colon, and prevent protection against pathogenic microbiota that may cause these gastrointestinal diseases (Piqué et al., 2019). *Bifidobacterium* and *Lactobacillus* are the main strains of probiotics that are used in the food industry, however certain species of *Streptococcus*, *Bacillus* and yeast such as *Saccharomyces* are also considered probiotics (Piqué et al., 2019).

According to Shori (2017), a sufficient amount of viable microorganism is required for food products, within the range of 10^6-10^7 colony-forming unit (CFU)/g, before ingestion to ensure that the product confers any health benefits. The nature of the probiotic, viability in the gastrointestinal tract and stability in food products poses a problem within food systems.

These microorganisms cannot survive the harsh conditions in the gastrointestinal tract as well as exposure to oxygen, which limits their functionality, and therefore, protection is required (Das et al., 2014). Microencapsulation is considered as one of the most efficient methods to confer protection of probiotics, preserving microorganisms in food and during the passage through the gastrointestinal tract allowing for controlled release, thus potentially contributing to the development of new functional foods. Other benefits include the simulation and production of secondary metabolites such as short-chained fatty acids which provide a wide range of physiological benefits to the host (Nazzaro et al., 2012). There are different methods of microencapsulation with the most common being extrusion, emulsion, spray drying as well as spray chilling. It is extremely important that the encapsulation technique does not reduce the probiotic survival rate and the probiotic activity by protecting and stabilizing the encapsulated component from harsh environmental conditions (Peanparkdee et al., 2016).

There are different types of material used as a coating membrane, such as polysaccharides use in conjunction with alginate, gelatine, carrageenan, and starch (Martin et al., 2015). The most appropriate membrane material is cost-efficient, readily or easily available, biocompatible, non-toxic or harmful and easy to handle (Ariful et al., 2010). The mechanism in which microencapsulation occurs is known as immobilization in which cells are entrapped within a matrix until their mobility is obstructed which restricts the microorganisms (Mitropoulou et al., 2013). There has been a recent trend in the use of prebiotics as a coating medium (Raddatz et al., 2020). Starch that resists digestion in the upper gastrointestinal tract is known as resistant starch and is also classified as prebiotic which stimulate the growth of beneficial bacteria in the colon. The objective of this study was to determine the viability and stability of *Bifidobacterium animalis* and *Lactobacillus casei* microencapsulated within a resistant starch medium. The resistant starch used for the microencapsulation process will be isolated from *Vigna unguiculata* and the microcapsules will be used for beverage application. Fruit juices are rich in nutrients, that do not contain any starter cultures therefore making it a good choice for the combination of probiotic microorganisms.

4.2. Materials and methods

4.2.1. Microencapsulation

4.2.1.1. Preparation of the bacterial suspension

Lactobacillus casei (ATCC 334) and *Bifidobacterium animalis* (ATCC 25527) were grown according to Zanjani et al. (2018). Cultures were activated in MRS broth at a temperature of 37°C under aerobic and anaerobic conditions respectively for 48 h. A concentration of Log 10 was obtained for each bacterial culture using the 0.5 McFarland standard and confirmed by checking the absorbance at 600 nm. Thereafter cells were harvested according to Seth et al. (2017) with modifications at 4000 *x g* for 10 min and rinsed twice in phosphate buffer saline (PBS) solution, with harvested cells used for microencapsulation.

4.2.1.2. Preparation of wall material and microencapsulation

All equipment used for preparation was sterilised at 121°C for 15 min. The preparation of wall material was done according to Ashwar et al. (2018) with minor modifications, briefly 10 g of resistant starch (isolated from cultivar *DT129-4* according to the method mentioned in 3.2.2) was added to 200 mL distilled water. The solution was heated until a gel was formed, then cooled to 30°C. Log 10/mL of each culture was separately added to the respective resistant starch solutions [RSL (resistant starch + *Lactobacillus casei*), RSB (resistant starch + *Bifidobacterium animalis*)] and both cultures added to another resistant starch [RSLB (resistant starch + *Lactobacillus casei* + *Bifidobacterium animalis*)] solution at a ratio of 1:1 (m/v). The solutions were then homogenised and freeze-dried at the temperature of −58.9°C under vacuum for 72 h (Tang et al., 2020).

4.2.2. Characterization of microcapsules

4.2.2.1. Particle size distribution

The particle size distribution of the isolated resistant starches was analysed using a laser light scattering particle analyser (Litesizer 500, Anton Paar., Graz, Austria).

4.2.2.2. Scanning electron microscope (SEM)

Microcapsules samples were mounted on stubs, coated with gold (10-20 nm in thickness) using a Quorum K150 RES Sputter Coater and viewed with a LEO 1450 SEM at an accelerating voltage of 5 kV.

4.2.2.3. Moisture content

Moisture was determined using the AOAC method 930.15. Briefly, samples were dried in an oven at 102°C for 3 h and moisture content measured.

4.2.2.4. Colorimetric analysis

Colour analysis of the microcapsules was measured using a colorimeter (ColorFlex EZ, Hunter Lab- SCFEZ 0840). The instrument was standardized with Hunter lab colour standards, and *L** (lightness), *a** (redness and greenness), and *b** (yellowness and blueness) values were measured.

4.2.3. Encapsulation yield after freeze-drying

According to Cruz-Benítez et al. (2019), 1 g of each microcapsule was mixed in 9 mL of 2% sodium citrate diluent, followed by shearing of the microcapsules using a vortex, thereafter 1 mL of the solution was mixed in 9 mL of peptone diluent. The colony-forming units (CFU g/mL) of cultures were determined by serial dilutions using MRS agar and incubation for 48 h at 37°C. The encapsulation yield calculated using the following equation:

$$\text{Encapsulation yield (EY) (\%)} = N/N_o \times 100$$

Where N is the log cell number (CFU) of viable entrapped cells released from the microspheres, and N_o is the log cell number (CFU) of free cells added to the wall material solution (Liao et al., 2018, Olivares et al., 2019).

4.2.4. Viability of microcapsules after simulated *in vitro* digestion

The simulated gastric juices (SGJ) and simulated intestinal juices (SIJ) were prepared according to Liao et al. (2017) and Moayyedia et al. (2018) with minor modifications. SGJ was prepared by dissolving sodium chloride (9 g/L) and pepsin (3 g/L) in deionized water and the pH adjusted to 2 using 0.1M HCl. Simulated intestinal juices were prepared by adding bile salts (6 g/L) and pancreatin (2 g/L) in a sodium chloride solution (9 g/L) and the pH adjusted to 6 using 0.1M NaOH, solutions were filter sterilized using a 0.22 µm filter. To determine the viability, 0.5 g of each microcapsule was added to 9 mL simulated gastric juice and simulated intestinal juice, respectively and incubated at 37°C under orbital shaking at 150 rpm for 60 and 120 min. After each incubation period viable bacteria were enumerated.

4.2.5. Viability and stability of microcapsules in fruit juice under cold storage

This was carried out according to Olivares et al. (2019), briefly 1 g of each microcapsule and 1 mL free cells were added to 100 mL of pasteurized juice and maintained under refrigeration conditions at 4°C. Samples were taken (after the microcapsules had settled) at 0, 7, 14, 21, and 28 d and colonies counted and expressed as Log10 CFU/mL of juice. After 28 d, the microcapsules were collected through centrifugation and enumerated by dissolving microcapsules in 9 mL of 2% sodium citrate diluent, followed by shearing of the microcapsules. Serial dilutions of the supernatant were then plated on MRS agar and aerobically incubated for 48-72 h at 37°C. The cultures were enumerated as CFU/g of spheres. For stability, samples were screened for pH levels and colour analysis at 0, 7, 14, 21, and 28 d.

4.2.6. Statistical analysis

Results are represented as mean±standard deviation for triplicate observations. Means were compared by two-way analysis of variance (ANOVA) using Tukey's Multiple Comparison test and confidence level of 0.05 (95% confidence interval).

4.3. Results and Discussion

4.3.1. Microencapsulation yield

Table 4.1 shows the efficiency of the encapsulation of *Lactobacillus casei* in resistant starch (RSL), *Bifidobacterium animalis* in resistant starch (RSB) and a combined *Lactobacillus casei* and *Bifidobacterium animalis* in resistant starch (RSLB). The encapsulation yield was found in the range of 81.55- 88.78% with RSLB having the highest yield. The initial microbial count in the resistant starch matrix before freeze-drying was between 10.10-10.18 Log CFU/mL and the yield of the microcapsules after freeze drying was in the range of 8.24-9.04 Log CFU/mL. Encapsulation yield is important to ensure the required number of probiotics are present as the encapsulation process affects the final amount of probiotics in the food or beverage product with the required amount for probiotics to be beneficial to the host being Log 6 (Afzaal et al., 2020). These results show that resistant starch has potential as a wall material for encapsulation.

Encapsulation yield is dependent on the type of wall material used and the method of encapsulation used (Zeashan et al., 2020). In a study by Prasanna and Charalampopoulos (2018) inulin, a prebiotic, was added to sodium alginate, with the sodium alginate and inulin mixture subsequently added to goats milk.

According to results obtained, adding prebiotics in the wall material can produce a higher yield. In another study by Poletto et al. (2019b) rice bran, inulin and resistant starch were added to alginate for the encapsulation of *Lactobacillus acidophilus* by external ionic gelation with the encapsulation yield ranging between 94.10-96.75%. The reasons for a slightly lower encapsulation yield in this study may be associated with the use of a resistant starch matrix without incorporating other materials. Most studies incorporate prebiotic material into wall materials such as sodium alginate, carrageenan and protein isolates to obtain a higher encapsulation yield. According Moayyedia et al. (2018), *Lactobacillus rhamnosus* was encapsulated in whey protein isolate, inulin and Persian gum matrices by electrospraying, freeze-drying and spray drying. Spray drying and freeze-drying were less detrimental to the microbial cells as compared to electrospraying, with freeze-dried microcapsules prolonged the survival of *Lactobacillus rhamnosus* during the transition in the digestive system.

Table 4.1: Encapsulation yield

Microcapsules	RSL	RSB	RSLB
Before drying (Log CFU/mL)	10.10 ± 0.09^{a}	10.12 ± 0.06^{a}	10.18 ± 0.09^{a}
After drying (Log CFU/mL)	8.24 ± 0.47^{b}	8.31 ± 0.38^{b}	9.04 ± 0.05^{a}
Encapsulation yield (%)	81.55 ± 4.4^{a}	82.16 ± 3.37^{a}	88.78 ± 0.99^{b}

All data are expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$). [RSL-resistant starch+*Lactobacillus casei*, RSB-resistant starch+*Bifidobacterium animalis*, RSLB-resistant starch+*Lactobacillus casei*+*Bifidobacterium animalis*]

4.3.2. Physicochemical analysis

4.3.2.1. Scanning electron microscope (SEM) and Particle size distribution

Figure 4.1 shows the scanning electron micrographs of all three microcapsules. There is no specific shape of the microcapsules, however, formed cluster matrices with some dents and cavities can be seen of the surface of the microcapsule. The red arrow shows the probiotic cells captured within the matrix. The morphology of the microcapsules is directly proportional to the materials and methods used in the encapsulation process (de la Cruz Pech-Canul et al., 2020). According to a study done by Moayyedia et al. (2018), similar results were obtained for the SEM images of freeze-dried microcapsules as the samples did not has a specific shape as well. The dents and cavities on the freeze-dried microcapsules may be due to the low drying temperatures, the mixed effect of atomization and the characterisation of the coat formation of the wall material used in the process (Moayyedia et al., 2018). Particle size analysis was also done.

The results for particle size varied amongst the microcapsules with the particle size being 19.63 μm for RSL, 20.90 μm for RSB and 9.58 μm for RSLB. Similar results were obtained in a study done by Ahmad et al. (2019) in which micro and nano-sized starch particles for encapsulation of camel milk-derived probiotics were compared. According to (Yao et al., 2020) 'microbial cells range between 1 to 10 μm whereas many colloidal delivery systems contain particles less than about 1 μm for example, microemulsions, nanoemulsions, and biopolymer nanoparticles.

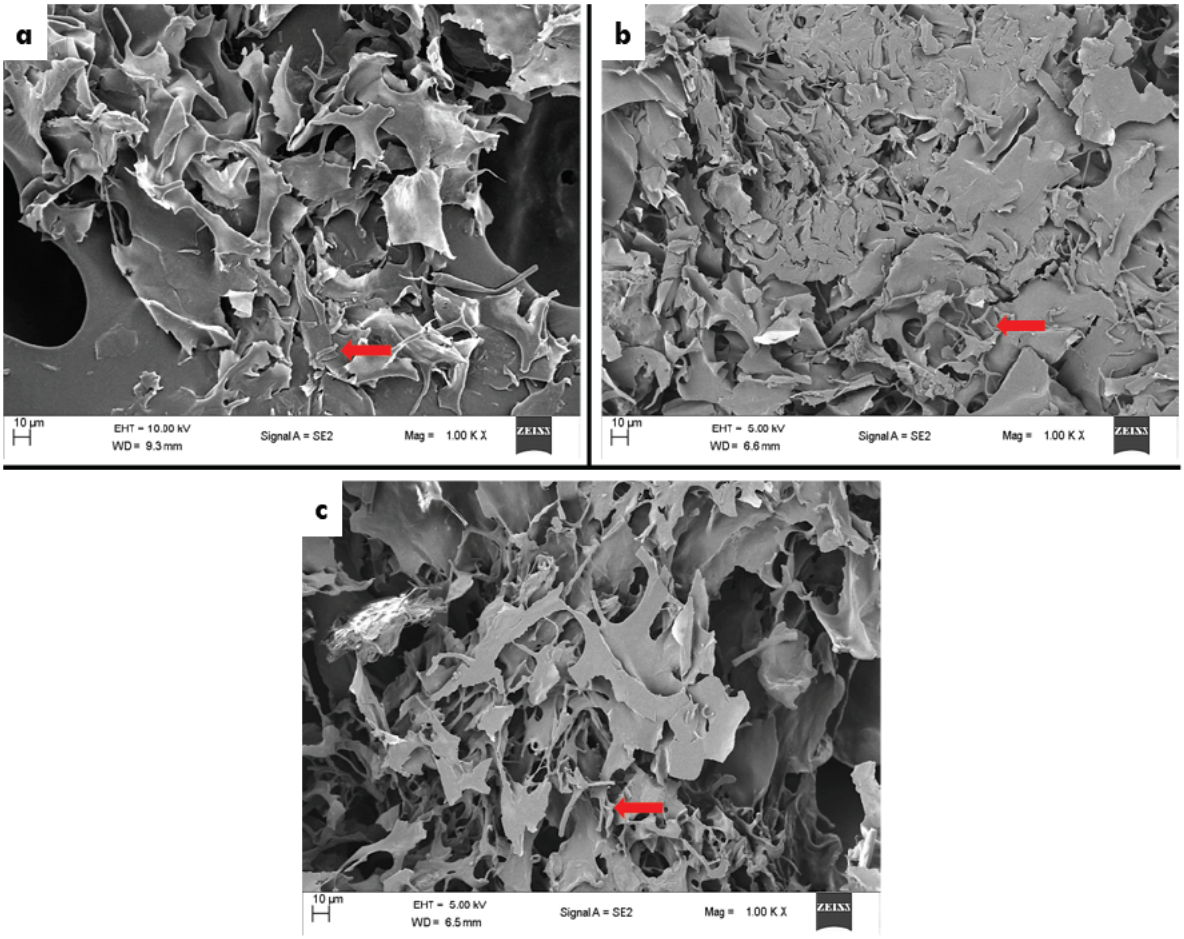

Figure 4.1: Scanning Electron Micrographs (SEM) of resistant starch microcapsules [a: RSL (resistant starch+*Lactobacillus casei*), b: RSB (resistant starch+*Bifidobacterium animalis*), c: RSLB (resistant starch+*Lactobacillus casei*+*Bifidobacterium animalis*)]. Red arrows depict probiotic cells captured within the resistant starch matrix (magnification: 1000x).

4.3.2.2. Colorimetric analysis and moisture analysis

Colour is an important aspect that attracts consumers to food products as colour is linked to flavour, nutrition and the quality of certain foods (Pieniazek and Messina, 2017). Colour analysis was done on the microcapsules as well as the resistant starch used as the wall material. According to Guo et al. (2019) "*L*" denotes the lightness ranging from 0 (black) to 100 (white), "*a*" denotes the redness represented by "+" to greenness which is represented "-" and "*b*" shows the yellowness represented by "+" to blueness represented by "-". *Whiteness* was calculated as: $100 - \sqrt{(100 - L)^2 + a^2 + b^2}$.

From the results shown in Table 4.3, the RS and the microcapsules were light in colour where *L**, a* and *b** were 75.01-83.31, 0.34-0.88 and 5.24-10.01 respectively. *Whiteness* ranged between 74.04-82.48. The RS sample being whiter as compared to the microcapsules, this could be due to discolouration that occurred during the encapsulation process. According to Pieniazek and Messina (2017), the heating process during the preparation of the wall material leads to a maillard reaction resulting in slight browning as well as the dehydrating procedures which affects the drying and rehydration kinetics may cause colour changes. Pieniazek and Messina (2017), also states that a higher moisture content promotes starch gelatinisation and development of darker pigments therefore the moisture content as shown in Table 4.2 could contribute for a discolouration. The results for moisture content were 23.03, 24.22 and 24.06% for RS, RSB and RSLB respectively, however, results for RS were 11.58%. In a similar study by Samedi and Charles (2019) where arrowroot starch was used for microencapsulation, the moisture content of the spray-dried samples were approximately 8%. In future, the moisture content can be decreased by increasing the time of freeze-drying as well as improving storage conditions.

Table 4.2: Moisture and colour analysis of microcapsules from *Vigna unguiculata* (*DTI29-4*)

	RS	RSL	RSB	RSLB
Moisture (%)	11.58±0.58^{b}	23.03±1.20^{a}	24.22±0.58^{a}	24.06±0.47^{a}
L*	83.31±1.23^{c}	77.38±0.02^{a}	75.03±0.01^{b}	78.21±0.01^{a}
a*	0.88±0.03^{a}	0.64±0.01^{c}	0.34±0.01^{d}	0.78±0.01^{b}
b*	5.24±0.05^{a}	9.7±0.01^{c}	7.08±0.00^{b}	10.01±0.01^{c}
Whiteness	82.48±1.16^{a}	75.38±0.02^{b}	74.04±0.01^{b}	76.01±0.01^{b}

All data are expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$). [RSL-resistant starch+*Lactobacillus casei*, RSB-resistant starch+*Bifidobacterium animalis*, RSLB-resistant starch+*Lactobacillus casei*+*Bifidobacterium animalis*]

4.3.3. Simulated Gastric Juice (SGJ) and Simulated Intestinal Juice (SIJ)

Viability of probiotics in the gastrointestinal tract is of vital importance to ensure that the required amount of probiotic reaches the colon and accomplishes its desired benefits (Zeashan et al., 2020). Figure 4.2 shows the effects of simulated gastric and intestinal juice on the microencapsulated probiotics and free cells when exposed for120 min. In SGJ, the FCL (free cells of *Lactobacillus casei*) decreased by 5.41 Log CFU/mL, FCB (free cells of *Bifidobacterium animalis*) decreased by 5.93 Log CFU/mL and FCLB (free cells *Lactobacillus casei* + *Bifidobacterium animalis*) decreased by 5.53 Log CFU/mL.

During the transition in SIJ, the FCL deceased by 5.45 Log CFU/mL, FCB by 5.95 Log CFU/mL and FCLB by 5.86 Log CFU/mL. This shows that the viability of free cells is significantly lower than the required amount for probiotics to provide any beneficial effects on the host. When the free cells are exposed to oxygen, gastric enzymes and gastric acid present in the gastrointestinal tract the viability is drastically affected, thus the importance of microencapsulation (Shori, 2017). The microencapsulated probiotics in this study had a substantial final count for SGJ and SIJ ranging between 7.30-7.98 Log CFU/mL and 7.26-7.89 Log CFU/mL respectively. This shows that the encapsulated probiotics remain viable after being exposed to both SGJ and SIJ for 120 min. The resistant starch therefore provided a level of protection for the probiotics from environmental stress in the GIT.

Since resistant starch is considered a prebiotic, when the microcapsules reach the colon, the probiotic bacteria present in the colon will feed on the prebiotics therefore the encapsulated probiotics will be released in the colon, stimulating an increase of good bacteria present in the colon therefore maintaining a healthy colon (Raddatz et al., 2020). Previous studies have shown that encapsulation of probiotics helps maintain their viability (Liao et al., 2017, Liao et al., 2018, Iqbal et al., 2018, Alfaro-Galarza et al., 2020). According to Moayyedia et al. (2018) variation in the viability of the probiotics in SGJ and SIJ may be due to the direct consequence of the applied methods. Similar results were obtained in a study done by Ashwar et al. (2018) in which RS was isolated from native rice starch and used as encapsulating material for *L. casei, L. brevis* and *L. plantarum*. In another study by Afzaal et al. (2019) probiotic bacteria were encapsulated in sodium alginate and carrageenan, found similar results for simulated gastrointestinal conditions.

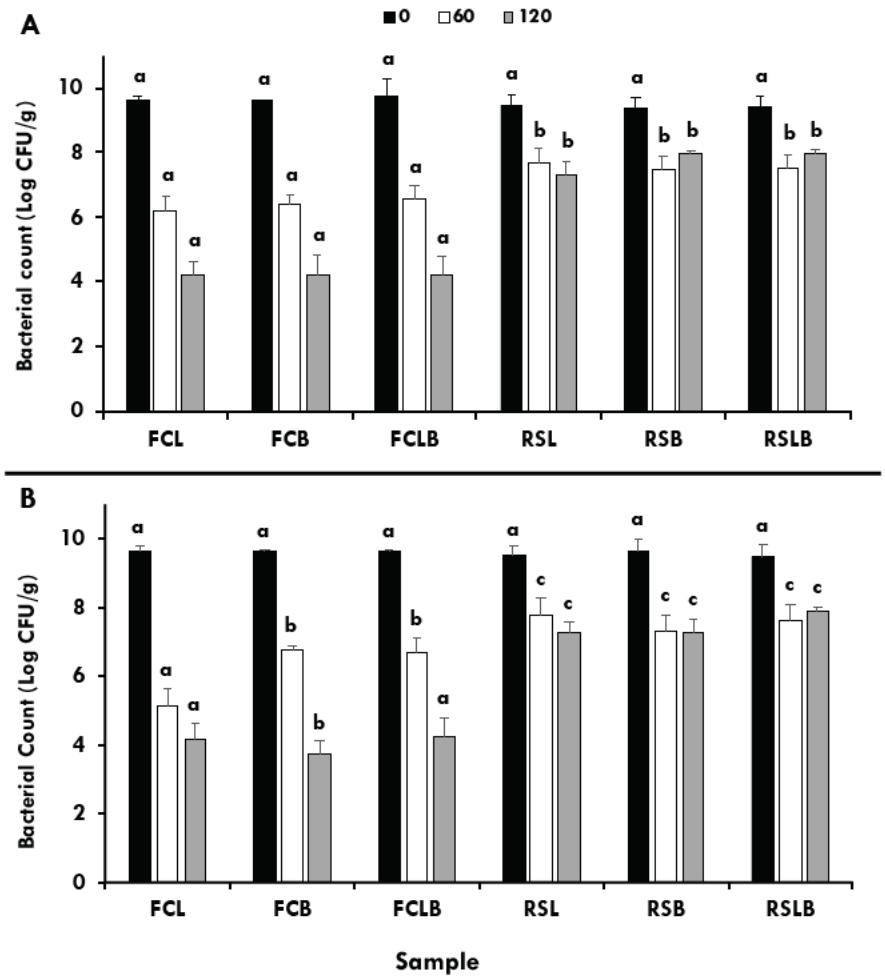

Figure 4.2: Viability of *Lactobacillus casei* and *Bifidobacterium animalis* in Simulated Gastric Juice (A) and Simulated Intestinal Juice (B) from 0-120 min. All data are expressed as mean±standard deviation (n= 3). Bars with different superscript letters are significantly different ($p<0.05$).

4.3.4. Stability and viability of microcapsules in juice

4.3.4.1. Colour analysis

Colour analysis of the juice application was done to determine the effect of the addition of the microcapsules on the colour of the final juice product. Plain apple juice, apple juice with free cells, apple juice with resistant starch and apple juice with microcapsules were compared (Table 4.3). Colour analysis is used to evaluate the overall quality and adequacy of the juice (Murtaza et al., 2020). From the result obtained each sample varied in relation to control juice sample. *Whiteness* for free cells was 2.44, 2.39 and 2.68 for FCL, FCB and FCLB respectively. The results for the RS and microcapsules RSL, RSB, RSLB were 13.71, 10.35, 9.73 and 7.94 respectively. The addition of the microcapsules significantly impacted the colour of the juice.

During visual inspection, the juice alone was golden yellow however when the microcapsules were added the juice became slightly darker in colour which validates the results obtained. Slight variation in colour amongst the microcapsules could be as a result of the dispersion of the microcapsules in the juice when the samples were analysed.

Table 4.3: Colour analysis of juice containing microcapsules and free cells

	Juice	RS	FCL	FCB	FCLB	RSL	RSB	RSLB
L*	1.34±0.09^{a}	13.78±0.05^{e}	2.50±0.24^{b}	2.47±0.38^{b}	2.71±0.04^{b}	10.42±0.27^{d}	9.75±0.04^{d}	7.96±0.04^{c}
a*	0.19±0.08^{a}	-0.18±0.04^{c}	0.15±0.02^{a}	0.10±0.01^{a}	0.02±0.05^{b}	-0.56±0.08^{e}	-0.67±0.08^{e}	-0.41±0.05
b*	-0.91±0.13^{a}	3.13±0.07^{d}	-1.26±0.05^{a}	-1.20±0.31^{a}	-1.42±0.07^{b}	1.20±0.09^{c}	1.20±0.09^{c}	0.94±0.12^{c}
Whiteness	1.30±0.03	13.71±0.02	2.44±0.21	2.39±0.34	2.68 ± 0.02	10.35±0.22	9.73±0.02	7.94±0.01

All data are expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$). [RS-resistant starch, FCL-free cells *Lactobacillus casei*, FCB-free cells *Bifidobacterium animalis*, FCLB-free cells *Lactobacillus casei*+*Bifidobacterium animalis*, RSL-resistant starch+*Lactobacillus casei*, RSB-resistant starch+*Bifidobacterium animalis*, RSLB-resistant starch+*Lactobacillus casei*+*Bifidobacterium animalis*]

4.3.4.2 pH analysis

Table 4.4 shows the pH of the juice over 28 d period, with samples analysed every 7 d. On day 0 the pH of the apple juice was 3.42 and, in the juice, containing RS the pH dropped to 3.34. The initial pH for the juice containing free cells ranged between 3.43-3.55 and the microcapsules ranged between 3.42-3.65. The pH for the apple juice remained constant over the 28 d period while there were slight fluctuations amongst the other samples. There was a decrease over the 28 days in the juice containing free cells and the juice containing microencapsulated cells which were found to be in the range of 3.41-3.42 and 3.25-3.37, respectively with a similar trend observed in a study done by Gandomi et al. (2016). In another study done by Afzaal et al. (2020) *Lactobacillus casei* was encapsulated in calcium alginate and whey protein and added to ice cream. Free and encapsulated cells were added to ice cream, and a decrease in pH was also observed in the results. According to Afzaal et al. (2020) addition of dietary fibre can increase the acidity within a sample therefore causing a drop in the pH. The RS which was used as the encapsulating material in this study is considered a prebiotic which is dietary fibre, therefore explaining the decrease in the pH.

Table 4.4: pH analysis of juice containing microcapsules and free cells

Day	Juice	RS	FCL	FCB	FCLB	RSL	RSB	RSLB
0	3.42±0.03^{a}	3.34±0.12^{a}	3.43±0.05^{a}	3.53±0.02^{a}	3.55±0.03^{a}	3.42±0.03^{a}	3.42±0.02^{a}	3.65±0.02^{b}
7	3.49±0.02^{a}	3.66±0.06^{b}	3.60±0.01^{b}	3.45±0.04^{a}	3.54±0.03^{a}	3.57±0.04^{a}	3.42±0.01^{a}	3.43±0.02^{a}
14	3.44±0.02^{a}	3.52±0.04^{a}	3.64±0.03^{b}	3.59±0.02^{b}	3.61±0.06^{b}	3.64±0.04^{b}	3.48±0.02^{a}	3.43±0.03^{a}
21	3.54±0.02^{a}	3.48±0.04^{a}	3.34±0.05^{a}	3.47±0.02^{a}	3.38±0.02^{a}	3.46±0.12^{a}	3.41±0.04^{a}	3.47±0.01^{a}
28	3.44±0.02^{a}	3.38±0.02^{a}	3.41±0.09^{a}	3.40±0.01^{a}	3.42±0.03^{a}	3.25±0.05^{a}	3.37±0.01^{a}	3.35±0.02^{a}

All data is expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$). [RS-resistant starch, FCL-free cells *Lactobacillus casei*, FCB-free cells *Bifidobacterium animalis*, FCLB-free cells *Lactobacillus casei*+*Bifidobacterium animalis*, RSL-resistant starch+*Lactobacillus casei*, RSB-resistant starch+*Bifidobacterium animalis*, RSLB-resistant starch+*Lactobacillus casei*+*Bifidobacterium animalis*]

4.3.4.3. Stability of microcapsules over 28 d

The stability of the capsules in low pH were analysed to determine the loss of probiotic cells from the microcapsules over 28 d (Table 4.5). Uninoculated juice, juice with resistant starch alone and juice with free probiotic cells served as a control samples in comparison to samples containing the microcapsules. The initial count for free probiotic cells at 0 d were 9.98, 10.17 and 9.70 Log CFU/mL for FCL, FCB and FCLB respectively. The final count at the end of 28 d for the free probiotic cells were 2.42, 3.52 and 3.08 CFU/mL for FCL, FCB and FCLB respectively with the amount required to be beneficial approximately Log 6 CFU/mL. This trend shows that free cells cannot survive in low pH environments, with a significant decrease in the free cell probiotic survival rate. Fruit juices are acidic which is harmful to many probiotic bacteria and numerous probiotic cells don't survive processing and storage in acidic environments (Srisukchayakula et al., 2018), with a study by Gandomi et al. (2016) displaying similar results. According to Olivares et al. (2019) probiotic bacteria are extremely sensitive to acidic conditions therefore encapsulation is necessary to ensure viability and stability to meet the requirements to confer health benefits. The stability of the capsules was evaluated by determining the probiotic release over 28 d. At 0 d there were no probiotics detected, however, at 7 d the results show that probiotic bacteria had been released from microcapsules, with the count being 2.28, 2.92 and 2.43 Log CFU/mL for RSL, RSB and RSLB respectively. With regard to stability at the end of the 28 d period, there was an overall increase in the number of probiotics that were released from the microcapsules into the juice samples with the count for RSL, RSB and RSLB being 3.84, 3.83 and 3.20 Log CFU/mL. According to Olivares et al. (2019), the carbohydrates present in the juice could stimulate the growth of probiotic bacteria that were released from the microcapsules.

Table 4.5: Stability of *Lactobacillus casei and Bifidobacterium animalis* in juice stored at 4°C over 28 d

Day	Juice	RS	FCL	FCB	FCLB	RSL	RSB	RSLB
0	0.00±0.00^{a}	0.00±0.00^{a}	9.98±0.05^{b}	10.17±0.05^{b}	9.70±0.63^{b}	0.00±0.03^{a}	0.00±0.00^{a}	0.00±0.00^{a}
7	0.00±0.00^{a}	0.00±0.00^{a}	5.10±0.01^{b}	7.17±0.73^{c}	5.14±0.59^{b}	2.28±0.04^{a}	2.92±0.57^{a}	2.43±0.16^{a}
14	0.00±0.00^{a}	0.00±0.00^{a}	3.70±0.03^{b}	3.63±0.08^{b}	4.65±1.04^{c}	3.17±0.04^{b}	2.56±0.09^{a}	2.56±0.09^{a}
21	0.00±0.00^{a}	0.00±0.00^{a}	2.41±0.05^{b}	3.50±1.36^{c}	4.21±0.53^{c}	3.61±0.12^{c}	3.14±0.54^{c}	3.13±0.53^{c}
28	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.09^{a}	3.52±0.38^{b}	3.08±0.38^{b}	3.84±0.05^{b}	3.83±0.43^{b}	3.20±0.47^{b}

All data is expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$). [RS-resistant starch, FCL-free cells *Lactobacillus casei*, FCB-free cells *Bifidobacterium animalis*, FCLB-free cells *Lactobacillus casei*+*Bifidobacterium animalis*, RSL-resistant starch+*Lactobacillus casei*, RSB- resistant starch+*Bifidobacterium animalis*, RSLB-resistant starch+*Lactobacillus casei*+*Bifidobacterium animalis*]

However, when the microcapsules were analysed for viability as shown in Table 4.6 the final yield for the microcapsules were 7.53, 6.98 and 7.46 Log CFU/mL for RSL, RSB and RSLB respectively. This confirms that microcapsules were considered stable during the juice application and that microencapsulation enhanced the survival of *Lactobacillus casei* and *Bifidobacterium animalis* in fruit juice under cold storage. The microcapsules provided a favourable anaerobic environment to the probiotic bacteria and acted as a physical barrier from the acidic condition associated with the fruit juice (Ding and Shah, 2008). In a study by Ding and Shah (2008) free and encapsulated probiotic bacteria were added to fruit juice to determining viability resulting in microencapsulated probiotics which were more stable and viable as compared to free probiotic cells.

Table 4.6: Viability of *Lactobacillus casei and Bifidobacterium animalis* microcapsules after 28 d

Viability	Juice	RS	FCL	FCB	FCLB	RSL	RSB	RSLB
Log CFU/mL	ND	ND	2.42±0.11^{b}	3.52±0.38^{b}	3.08±0.38^{b}	7.53±0.47^{a}	6.98±0.87^{a}	7.46±0.32^{a}

All data is expressed as mean±standard deviation (n= 3). Values with different superscript letters are significantly different ($p<0.05$). [RS-resistant starch, FCL-free cells *Lactobacillus casei*, FCB-free cells *Bifidobacterium animalis*, FCLB-free cells *Lactobacillus casei*+*Bifidobacterium animalis*, RSL-resistant starch+*Lactobacillus casei*, RSB- resistant starch+*Bifidobacterium animalis*, RSLB-resistant starch+*Lactobacillus casei*+ *Bifidobacterium animalis*]

4.4 Conclusion

Microencapsulation of probiotics is extremely important to ensure that the cells are protected under harsh environmental conditions. Encapsulation safeguards the viability and stability of probiotic cells in the gastrointestinal tract and when used in food or beverage application. The results obtained in this study was highly favourable given that resistant starch was used on its own. Resistant starch has great potential as an encapsulating material for probiotics as it produced a considerably high encapsulation yield freeze-drying. Further studies can be done on the use of resistant starch combined with other encapsulating agents as well as using resistant starch encapsulated probiotics in other food or beverage application to determine viability and stability for other potential food applications.

Chapter 5: General Discussion

Probiotics have a wide range of health benefit such as they maintain a healthy colon by improving digestion and nutrient absorption, and have anti-inflammatory, anti-carcinogenic, anti-infection, antimutagenic and aids in the prevention of diarrhoea and constipation (Pandey et al., 2015). Microencapsulation of probiotics is extremely important to preserve the probiotic in the GIT until it reaches the colon to confer a health benefit to the host (da Silva et al., 2014). Different factors need to be taken into consideration during the microencapsulation process such as the probiotic that will be encapsulated, the encapsulating material and the method that will be used for encapsulation. These factors play a major role in the result and efficiency of the microcapsules (Frakolaki et al., 2020).

In this study, the probiotic microorganisms used was *Lactobacillus casei* and *Bifidobacterium animalis* as they are common probiotic microorganisms used in encapsulations (Yao et al., 2020). As mentioned previously, for microencapsulation to be effective, a concentration of Log 6 and above is required (Liu et al., 2020). In this study resistant starch was used as an encapsulating membrane. The resistant starch was isolated from various cowpea cultivars. Cowpea is an underutilized indigenous crop native to Africa and is highly nutritious (Oyeyinka et al., 2020). Resistant starch isolation, structural and physicochemical analysis was done on 5 cultivars of cowpeas (Bechuana white, TVU 11424, PAN 311, Fahari, DT129-4), the one with the most favourable results and the most potential to be used as an encapsulation material, was cultivar DT129-4 as it yielded 13.74 % resistant starch, the highest amount as compared to the other cultivars.

Resistant starch yield is affected by granular morphology such as size, shape and surface area of the granules. Smaller granules are more exposed to enzymatic and acidic hydrolysis as compared to larger granules and crevices on the surface of the granule can allow easy penetration of enzymes and acids. Amylose and amylopectin ratio and starch source are also factors affecting starch digestion. High amylose lowers the starch digestibility (Zaman and Sarbini, 2015, Jyothsna and Hymavathi, 2017, Tacer-Caba and Nilufer-Erdilb, 2018). The structural analysis done was SEM, ATR-FTIR and XRD which showed that cultivar DT129-4 has a stronger crystalline structure as compared to the other cultivars. The physicochemical analysis done was colour analysis, amylose content, water and oil capacity, foaming and emulsion stability and capacity as well as swelling and solubility, the results obtained for DT129-4 was highly favourable.

The results for foaming and emulsion was significantly low as it is dependent on the amount of protein present (Wani et al., 2015) and due to the sample being a starch isolate the results were expected. Physicochemical properties are analysed to determine the interaction between food components for the utilisation of a sample in food and beverage application (Naiker et al., 2019).

Resistant starch is also known to have prebiotic potential which can stimulate the growth of beneficial bacteria present in the colon (Topping et al., 2003, Bird et al., 2010, Gibson et al., 2017). Resistant starch is considered a potential material for encapsulation due to its non-digestible characteristic in the upper gastrointestinal tract therefore making it an appropriate protective material for the probiotic microorganisms (Tacer-Caba and Nilufer-Erdilb, 2018). In this study, the resistant starch from cultivar DT129-4 was heated to form a gel, when the gel was cooled the probiotics were added and homogenised so that the probiotic cells would be attached and confined within the resistant starch matrix. The encapsulation yield was found between 8.24 -9.04 log CU/mL, fulfilling the desired amount to be beneficial. A loss in yield could be due to the transition into the resistant starch matrix as well as the freeze-drying process. Enzymes present in the gastrointestinal tract such as salivary and pancreatic enzymes as well as the acidic conditions in the stomach affects the probiotics (Bhattarai et al., 2017)

The microcapsules and free cells were exposed to simulated gastric and simulated intestinal juice to ensure that the required amount is viable after 120 minutes under gastrointestinal conditions. The microencapsulated probiotics in this study had a substantial final count for SGJ and SIJ ranged between 7.30 – 7.98 Log CFU/mL and 7.26 – 7.89 Log CFU/mL respectively. The Log CFU/mL for free cells were significantly lower than the microcapsules. The microcapsules were then added to apple juice to determine the stability and viability of the microcapsules after 28 days under cold storage. Colour analysis was done to determine the colour variation when microcapsules were added to the juice. Visually the colour of the juice had a slight change when the capsules were added. The degree of whiteness of the juice alone was 1.30 which confirmed the dark golden colour, however, the degree of whiteness was 10.35, 9.73 and 7.94 for RSL, RSB and RSLB respectively.

The analysis of pH was done to determine the stability of the juice containing the microcapsules. There were slight fluctuations in the pH over the 28 days with the overall pH ranging between 3.25-3.37 for the probiotic enriched juices. The addition of dietary fibre i.e. resistant starch can decrease the pH (Afzaal et al., 2020). Viability of the microcapsules in the juice yielded a final count of 7.53, 6.98 and 7.46 Log CFU/mL for RSL, RSB and RSL respectively. The results for the combined microcapsules (RSLB) displayed favourable results and This confirms he main aim of this project, that the microencapsulation enhanced the survival of *Lactobacillus casei* and *Bifidobacterium animalis* in the gastrointestinal tract and the fruit juice under cold storage.

Conclusions and future recommendations

The research work presents a practical application in using resistant starch only as an encapsulating material. This study also encourages the use of microencapsulated probiotics to enhance the viability and stability in the gastrointestinal tract as well as in other varieties of food and beverage. There is great potential for microencapsulated probiotics in the food industry as well as the pharmaceutical industry. These microcapsules can satisfy the consumer-driven demand for probiotic-based healthy products, however, further studies can be done to improve the viability and stability of microcapsules

- Resistant starch isolated from other sources such as green banana, potatoes and various legumes.
- Resistant starch can be used in combination with other suitable wall material to enhance the viability and stability of the microcapsules as well as the use of different encapsulating methods can be explored
- Microcapsules can be added to various food and beverages such as baked goods, gummy sweets, chocolates, breakfast cereals etc.

Chapter 6: References

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