THE EFFECT OF SOLID-STATE FERMENTATION ON AIR-CLASSIFIED PEA PROTEIN-ENRICHED FLOUR TO IMPROVE THE DIGESTIBILITY AND FUNCTIONAL PROPERTIES

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ABSTRACT

The overarching goal of this research was to investigate the effect of solid-state fermentation (SSF) on pea protein-enriched flour (PPEF) to improve the digestibility and functional properties. The pea protein is classified as an enriched flour since it has a higher protein content than pea flour (20-30%) however, is less than a concentrate (70%) or isolate (90%) (Singhal *et al.*, 2016; Lam *et al.*, 2018). PPEF was inoculated with *Aspergillus oryzae* NRRL 5590 or *Aspergillus niger* NRRL 334 and evaluated at two temperatures (30°C and 40°C) over 48 h of fermentation to obtain limited protein hydrolysis (0-10%). Limited protein hydrolysis was acquired to enhance functionality properties in addition to the protein digestibility. The surface charge of fermented PPEF increased in negativity over fermentation time for both fungi: from -16.2 to -18.4 mV for *A. oryzae* and from -13.5 to -18.6 mV for *A. niger*. Whereas, surface hydrophobicity decreased from 14.1 to 8.4 a.u. for *A. oryzae* and 21.6 and 13.9 a.u. for *A. niger*. Fermented PPEF was analyzed for nitrogen solubility, emulsifying and foaming properties at pH values of 3.0, 5.0 and 7.0. In all samples, functionality (based on solubility, emulsification and foaming) was found to be greatest at pH 3.0 and 7.0 and lowest at pH 5.0 (near the isoelectric point of PPEF). Specifically, fermented PPEF was found to significantly decrease in solubility over fermentation time for both fungi at all pH values tested ($p<0.001$). Due to the low solubility in all fermented samples, the functional properties (foaming and emulsifying properties) that are dependent upon high solubility were negatively impacted. However, water and oil holding capacities of fermented PPEF increased over the 6-h of fermentation. Water holding capacity increased from 1.5 g/g to 2.0 g/g with *A. oryzae* fermentation, and oil holding capacity increased from 1.2 g/g to 2.3 g/g with *A. niger* fermentation.

Fermentation was found to improve protein quality of PPEF. Proteolysis inhibition, i.e., activities of trypsin and chymotrypsin inhibitors, was found to reduce over fermentation (~11-30% and 22-23% in *A. oryzae* and *A.niger*, respectively). Whereas, total phenolic content was shown to increase during fermentation (from ~38-44%). *In vitro* protein digestibility showed an increase over the fermentation time, from 6-8%, and could be attributed to reduced activity levels of enzyme inhibitors. *In vitro* protein digestibility corrected amino acid score showed a reduction of ~5-15%

at 6 h, with limiting amino acids remaining methionine and cysteine. Together, these findings suggest that SSF of PPEF could potentially improve digestibility through the reduction of bioactive properties and overall improvement of protein quality. Since SSF with *A. niger* and A. *oryzae* led to poor protein quality, it is not recommended as a means for altering the nutritional value of pea protein enriched flour. Overall, SSF is an efficient method for improving the oil and water holding capacities and protein digestibility in PPEF.

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LIST OF ABBREVIATIONS AND SYMBOLS

A. oryzae	*Aspergillus oryzae*
A. niger	*Aspergillus niger*
a.u.	Arbitrary unit
ANOVA	Analysis of variance
ANS	8-anillino-1-naphthalenesulfonic acid
CIA	Chymotrypsin inhibitor activity
CIU	Chymotrypsin inhibitory unit
d.b.	Dry weight basis
DH	Degree of hydrolysis
DIAAS	Digestible indispensable amino acid score
EA	Emulsion activity
ES	Emulsion stability
FAO	Food and agriculture organization
FC	Foaming capacity
FS	Foaming stability
GAE	Gallic acid equivalent
IVPD	*In vitro* protein digestibility
IV-PDCAAS	*In vitro* Protein Digestibility Corrected Amino Acid Score
OHC	Oil holding capacity
PDA	Potato dextrose agar
pI	Isoelectric point
PPEF	Pea protein-enriched flour
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SmF	Submerged fermentation
SSF	Solid-state fermentation
TIA	Trypsin inhibitor activity
TIU	Trypsin inhibitor unit
TPC	Total phenolic content
ZP	Zeta potential
g	Gravitational force

1. INTRODUCTION

1.1 OVERVIEW

Field pea (*Pisum sativum L.)* is a low cost, abundant crop which, when fractionated, offers various beneficial applications to the food industry. A common method of fractionation is air classification. It separates milled flour into two major fractions: light and heavy fractions. The heavy fraction is mainly composed of starch, and the light fraction is rich in proteins. The light fraction is often referred as pea protein-enriched flour. Pea protein-enriched flour (PPEF) is composed of approximately 40-60% protein content, making its content greater than flour (20-30%) but less than protein concentrates and isolates. PPEF have the potential to serve as alternatives to animal-derived protein and a complementary protein to cereal crops. Pea is composed of 21-25% of protein and contains essential amino acids necessary for human health (McKay *et al.*, 2003; Boye *et al.*, 2010b). Specifically, pea protein contains high levels of lysine and threonine, that are generally low in cereal grains (Oelke *et al.*, 2000). Whereas, pea protein is relatively low in tryptophan and sulfur containing amino acids such as, methionine and cysteine (Swanson, 1990). Therefore, pea protein is a complementary protein in which, consumption of pea protein and cereal grains should be done together to obtain adequate essential amino acids. Saskatchewan is the largest producer of pea in Canada, therefore they are a valuable export crop for either animal and human consumption (Pulse Canada, 2016). Protein is an important part of the human diet; protein is nutritionally required for the growth and repair of tissue as well as the overall health and well-being of humans. In addition to their nutritional benefits, proteins play functional and structural roles in foods which make them desirable to consume. As the population of the world increases, so will the demand for protein.

In developed countries, awareness and demand for healthier plant-based food sources is increasing due to their beneficial protein content and sustainability (Chandra-Hioe *et al.*, 2016). As consumer awareness and acceptance for healthy, sustainable alternatives to meat products increases, plant-based protein products can fulfill this demand. In developing countries, PPEF as a food ingredient is advantageous due to its dense nutrition composition and functional properties, their availability and affordability to developing countries' economy. Pea protein is a promising

replacement of currently-commercialized soybean due to its non-GM status and low allergenic properties (Meinlschmidt *et al.*, 2005).

Field pea is extremely beneficial to the human diet as it contains bioactive compounds. Bioactive properties are linked to health benefits such as anti-cancer properties and antioxidant activity to reduce free radicals (Singh and Basu, 2012). However, the presence of bioactive compounds such as, protease inhibitors can decrease protein bioavailability and lower the nutritional value due to inhibiting complete protein digestibility (Sandberg, 2002). Thus, bioactive compounds can affect the overall protein digestibility and quality. To improve the nutritional profile of pea proteins, reduction or elimination of the inhibitory activity through processing can aid to improve the nutritional profile of PPEF. In addition, it is beneficial for marketability and range of applications of PPEF.

The processing method of SSF involves the growth of microorganisms on moist substrates in the absence of free-flowing water (Falony *et al.*, 2006). It is an emerging method to modify legumes to improve their overall functional and nutritional properties (Chawla *et al.*, 2017). Through fermentation the alteration of compounds can occur, producing shorter chain compounds with lower molecular mass (Xiao *et al.*, 2015). Fermentation can also help to reduce bioactive compound activity in flour. Accordingly, this project seeks to investigate fermentation as a means to improve digestibility, nutritional and functional properties of PPEF; particularly, to optimize SSF to improve the digestibility and functionality of pea protein.

1.2 OBJECTIVES

The overarching goals of this research project are to employ fungal solid-state fermentation (SSF) to modify pea protein-enriched flour (PPEF) and evaluate its effect on the protein's physicochemical, functional and protein digestibility properties. This study will provide new insights into the effects of SSF as means of improving PPEF for human consumption. The specific objectives of this research include:

- To measure the impact of fermentation conditions (time, pH and temperature) on the degree of hydrolysis of PPEF.
- To examine the effect of pH on the surface properties and functionality properties of PPEF.
- To determine the impact of SSF on bioactive compounds of PPEF.

- To determine the impact of SSF amino acid profiles, *in vitro* protein digestibility and protein quality of PPEF.

1.3 HYPOTHESS

To achieve the overall goal of this research project, the following hypotheses were tested:

- Greater degree of hydrolysis of PPEF will be found at longer fermentation periods, where more microbial growth in conjunction with microbial metabolic activity will lead to greater unraveling of protein structures.
- Increased fermentation times will result in both an increase in surface charge and hydrophobicity, as it is presumed more of the protein will become unraveled, thereby exposing buried reactive groups.
- Fermentation will result in increased oil and water holding capacities, caused by a greater number of reactive groups becoming exposed. The effects will be greater at pH values further from the protein's isoelectric (pI) point (i.e., pH 3.0 and 7.0).
- Foaming, emulsification and solubility will decrease with fermentation due to a rise in hydrophobicity of the fermented PPEF. The aforementioned functional properties will be improved at pH values further from the pI value (i.e., 3.0 and 7.0).
- Levels of bioactive properties will decrease as fermentation time increases.
- Protein digestibility will increase as fermentation time increases.

2. LITERATURE REVIEW

2.1 Pea protein structure

Pea is one of the major food legumes grown in the world, ranking fourth in global production below soybeans, peanuts and dry beans and are grown on over 25 million acres (10.1 million hectares) worldwide (McKay *et al.*, 2003). The appeal of pea protein has much to do with their potential as an alternative to soybean protein (Vidal-Valverde *et al.*, 2003). The five main types of pea grown worldwide are Austrian winter pea, green pea, maple pea, marrowfat pea and yellow pea. More than 60 varieties of pea have been developed by crop scientists in Canada (Roy *et al.*, 2010). The protein content of pea ranges from 23.1% to 30.9%, depending on cultivation conditions (Lam *et al.*, 2018). The storage proteins found within the pea include the water-soluble albumins and the more abundant, salt-soluble globulins. Globulins consist primarily of vicilin (7*S*) and legumin (11*S*) proteins In pea protein, globulins account for 70-80% and a minority fractions: albumins (10-20%) of the total protein (Lam *et al.*, 2018). Albumin proteins are composed of enzymes, protease inhibitors, amylase inhibitors, and lectins. Albumin proteins are comprised of two major fractions, a larger albumin fraction and a minor one (Lam *et al.*, 2018). The remaining pea protein is made up of minor amounts of prolamins that are soluble in dilute alcohol and glutenins that are soluble in dilute acid (Lam *et al.*, 2018). Pea protein is rich in most of the essential amino acids, particularly tryptophan and lysine (Roy *et al.*, 2010). Albumins contain more of the essential amino acids (e.g., tryptophan, lysine, threonine, cysteine and methionine) in comparison with globulin. Pea globulin proteins are rich in arginine, phenylalanine, leucine and isoleucine (Boye *et al.*, 2010b). Meanwhile, pulse storage proteins are relatively low in tryptophan and sulfur-containing amino acids, such as methionine and cysteine, but contain higher lysine content compared to cereal crops. For example, pea protein consists of 20-30% lysine-rich protein (Swanson, 1990). Therefore, consumption of pea proteins should be ideally done in unison with methionine and cysteine-rich foods such as, cereals, to obtain optimal nutrition.

2.2 Protein extraction and fractionation

The extraction procedures affect characteristics and applications of pea protein. The yield and purity are notably affected by extraction. Therefore, employing the proper protein extraction methods is important to ensure successful separation of protein from seed and sufficient recovery of the protein. More importantly, the technique used determines the proteins functionality such as, emulsification, solubility, foaming, fat-binding, water-binding, gelation, swelling, viscosity and physicochemical properties (Can Karaca *et al.*, 2011). Alkaline extraction followed by isoelectric precipitation and salt extraction are the common methods performed in the extraction of legume protein isolate to ensure a wide range of functionality in isolate proteins. Whereas, legumin protein concentrate and legume starch are separated through air classification. Extractions are favorable due to their improvement of nutrition and health, removal of undesirable flavour compounds and functionality such as, heat stability (Boye *et al.*, 2010b).

2.2.1 Air classification

Air classification is a procedure to separate protein from starch. This process is a dry fractionation process, costing less than wet extraction processes due to the money saved on not having to dry the fractions after processing (Coda *et al.*, 2015). The seed is ground into a fine flour and classified in a spiral air stream which fractionate protein and starch based on size, shape and density of the particles. Air classification is used to separate milled pea and lentil into a light or fine fraction, i.e., protein concentrate, and a heavy or course fraction, i.e., starch concentrate (Swanson, 1990). The process can be repeated to increase the efficacy of protein and starch separation. In comparison to aqueous extraction processes, the two air classification fractions do not attain as high of purity due to cross contamination of fractions with one another (Boye *et al.*, 2010b).

Air classified pea and lentil protein concentrates normally contain 38-65% of protein. Air classification is a well-adapted process in the extraction of pea flours due to the large diameter and uniform distribution of starch granules found in legumes (Swanson, 1990). Coda *et al.* (2015) found air classification extraction, when combined with fermentation, was a beneficial process to increase the quality of the protein of faba bean flour. They evaluated the effect of air classification and fermentation by *Lactobacillus plantarum* VTT E-133328 on faba bean flour in order to remove anti-nutritional properties and improve the nutritional properties. The results of the study showed

that the combination of air classification and fermentation improved nutrition and functional properties of faba bean flour, which could then be implemented into various food products. As well as, the process increased the digestibility of faba bean protein matrices. Air classification allowed the separation of the flour into the protein and starch-rich fractions. The process separated the anti-nutritional factors mainly from the protein-rich fractions and lowered the amount of anti-nutritional factors in the starch-rich fraction. The authors concluded that the major advantages of air classification are: the small changes in the chemical properties of the flour components by removing the anti-nutritional factors, the preservation of functional properties of the fractions, and the lower energy input needed in comparison to wet fractionation. Additionally, the combination of air classification and fermentation was a favorable method for improving free amino acids content, increasing the protein digestibility and diminishing anti-nutritional factors (Coda *et al.*, 2015).

2.2.2 Alkaline extraction

Alkaline extraction combined with isoelectric precipitation is a common pea protein extraction method (Boye *et al.*, 2010a). Alkaline extraction/isoelectric precipitation is employed by using the solubility of proteins as an advantage. At a high alkaline pH and low pH values close to their isoelectric point (~pH 4-5) pea protein is precipitated out of the crude flour (Boye *et al.*, 2010a). Ground pulse flour is dispersed in water resulting in a flour suspension ratio ranging from 1:5 to 1:20. Following the flour suspension, the pH is adjusted to alkaline (pH 8-11) and the mixture is left to stand for 30-180 minutes to ensure solubilization of the protein. During this step the mixture remains at an alkaline pH, and the temperature can be increased (55-65ºC) thereby improving the solubilization and extraction of the protein. Afterwards, the mixture is filtered to remove insoluble material. In addition, the pH is adjusted to the isoelectric point to induce protein precipitation. The last steps include centrifugation to recover the protein, washing to remove salt, neutralization to ensure greater performance of protein and drying (Boye *et al.*, 2010a). Swanson (1990) studied pea and lentil protein extraction and its effect on functionality, and found that pea isolates prepared using an alkaline process contained 90-95% protein with an overall protein yield of 80%. Alkaline extraction of proteins causes several adverse chemical reactions, including: racemization of amino acids, reduced protein digestibility, and loss of essential amino acids such as cysteine and lysine (Swanson, 1990).

2.2.3 Acid extraction/isoelectric precipitation

Acid extraction is similar in procedure to that of alkaline extraction. However, the major difference is that the initial protein extraction is performed under acidic conditions. Solubility of pea protein is greater under acidic conditions (pH<4), where in the low pH solubilizes the protein before precipitation. If the pH is too acidic the protein can be damaged and result in less-efficient protein recovery. Therefore, acid extraction processes have difficulty in precipitating proteins and thus tend to not recover as much protein as alkaline-based extraction methods (Boye *et al.*, 2010a). Can Karaca *et al.* (2011) evaluated isoelectric precipitation and salt extraction's effect on emulsification in the production of chickpea, faba bean, lentil and pea proteins. The study found that isoelectric precipitation resulted in isolates with higher surface charge and solubility compared to salt extraction. The authors indicated that the extraction method has a great effect on the protein functionality due to the alteration of globulin/albumin or legumin/vicillin ratios and the physicochemical characteristics of the protein (Can Karaca *et al.*, 2011).

2.2.4 Water extraction

Water extraction is performed by simply using cold water combined with crude flour and omitting the acid precipitation step, thereby removing water-soluble proteins. This is a less-harsh method due to the exclusion of acid precipitation treatment. In order to increase the recovery of protein, the water extraction can be completed twice. The extracted albumin protein isolate is the end product through water extraction (Boye *et al.*, 2010a).

2.2.5 Salt precipitation extraction (micellization)

Salt extraction is salting-in and salting-out of protein to remove the protein from the crude flour. Salting-out is highly-dependent on the surface hydrophobicity of the protein, as well as the pH and temperature which also influence protein precipitation. After extraction of protein using an appropriate salt solution at the desired ionic strength, the solution is diluted, inducing protein precipitation that can then be recovered by centrifugation or filtration, followed by drying. At high salt levels, protein solubility decreases, therefore salting-out competes with the protein for water. Salt-water interactions are favored over protein-water interactions, causing the disruption of the protein's hydration layer and exposing hydrophobic patches on the protein. Sun *et al.* (2010) evaluated the effect on gelation properties when extracted by salt-extraction. The salt extraction

caused minimal protein denaturation making its functionality favourable. The pea protein extracted by salt extraction showed superior gelation ability in comparison with samples prepared by isoelectric precipitation and spray drying (Sun *et al.*, 2010).

2.2.6 Ultrafiltration

Ultrafiltration process uses supernatant obtained after alkaline or acid extraction and then filtrated to concentrate the proteins. Separation of proteins in solution is based on molecular size. This process is a gentle process due to no heat application; it is generally used as an alternative to isoelectric precipitation. Boye *et al.* (2010a) evaluated ultrafiltration and isoelectric extraction techniques on the functionality properties of pea, chickpea and lentil protein concentrates. Ultrafiltration concentrates were higher in protein content (69.1-88.6% w/w) when compared to isoelectric precipitation (63.9-81.7% w/w). Additionally, the extracted protein had good functionality properties such as, solubility, fat binding capacity, emulsifying stability and foaming properties (Boye *et al.*, 2010a).

2.3 Fermentation

Fermentation is the biological conversion of complex substrates into simple compounds through the use of microorganisms, that include bacteria and molds, via their enzymes and metabolic products (Subramaniyam & Vimala, 2012). This processing method has been used for thousands of years. Notable examples of food fermentation include *Aspergillus oryzae* fermentation of rice for koji production, and *Penicillium roqueforti* used for the production of blue cheese (Holker *et al.*, 2004). Fermentation is an ancient process which has been modified and improved throughout history to maximize production and overall efficiency. More recently, fermentation has seen increasing application for improving the nutrient content and digestibility of various foods. The process of fermentation has been demonstrated to not only improves the nutritional value and digestibility, but also enhance flavour, colour, functional properties, while decreasing allergenicity and anti-nutritional factors. The nutritional features that fermentation can aid include increased production of bioactive compounds and essential amino acids, increased vitamin and mineral content, and improved carbohydrate availability (Sanjukta & Rai, 2016).

2.3.1 Two types of fermentation

There are two types of commercial fermentation, based on the type of media used: solid-state fermentation (SSF) and submerged fermentation (SmF). SSF involves the growth of microorganisms on water-insoluble substrates in the absence of free water (Hamidi-Esfahani *et al.*, 2004). SmF processing is based on the growth of microorganisms in a free-flowing liquid medium, where the liquid contains the nutrients required for microbial growth. Both SSF and SmF methods are dependent on the selection of the microorganism used in the process for the best productivity.

In SSF the substrate has to retain a sufficient amount of moisture to support the growth and metabolism of the microorganism in order for fermentation to proceed. Furthermore, the natural habitat of the microorganism should be similar to the environment created in SSF, making it a preferred choice for the growth of the microorganism. Thus, SSF is optimal for fermentation by fungi and microorganisms that require low moisture contents since there is a low moisture content within a solid-state closed system. Conversely, microorganisms that require high water activity, such as bacteria, are not well-suited for SSF. In general, substrates are utilized more slowly and steadily during SSF therefore, the same substrate can be used for long or extended fermentation periods (Subramaniyam & Vimala, 2012). Microbes of interest will grow and utilize the moist substrate materials in the absence of free water. Low humidity in SSF makes microbes more capable of producing certain enzymes and metabolites, which usually will not be produced in a SmF (Viniegra-Gonzalez *et al.*, 2003). A higher growth rate by fungi on solid substrate was determined in SSF, in contrast with SmF. This is due to the morphology of the filamentous fungi which allows the fungi to colonize the surface while hyphae penetrate into the solid matrix, while simultaneously-secreting enzymes and metabolites, thereby extracting nutrients for growth. SSF is an advantageous method to use to process feed, fuel, food, industrial chemicals and pharmaceutical products, due to its low-cost of producing the product, the simplicity of operation and high-volume production (Pandey, 2003; Novelli *et al.*, 2016). However, there are disadvantages to SSF since it is sometimes difficult to maintain constant or adequate moisture content, distribute aeration uniformly, maintain purity of inoculum and fermentation cultures, and remove metabolic heat (Wang *et al.*, 2005). Therefore, scaling-up of production, and maintaining efficient operation, are sometimes extremely difficult to implement.

Alternatively, SmF has become well-established over the past century in comparison to SSF. SmF offers ease of process control making it simpler, in addition to reducing fermentation

times. This process, while historically used to ferment alcoholic beverages and dairy products, has also seen application for the production and extraction of bioactive compounds. In SmF, substrates are utilized rapidly therefore constant replacement of nutrients is needed. The SmF environment, in contrast to that of SSF, is considered to violate some microorganism's natural habitat because of the high-water concentration, especially in the case of fungi. Therefore, SmF is preferred for bacteria that require high moisture content (Subramaniyam & Vimala, 2012). SmF is easily adapted for industrial applications and has an obvious advantage when scaling-up production, since parameters such as temperature and moisture, are easier to control in comparison to SSF (Couto & Sanroman, 2006).

2.3.2 Fungal fermentation of legumes

Fungal fermentation of legumes potentially increases the nutritional value of the substrate, the amount of antioxidants, as well as yield vitamins and compounds that improve digestibility. The process of fermentation can also eliminate anti-nutritional factors present in seeds and thus provides an alternative to other treatments, such as long-duration soaking or cooking of seeds. Particularly beneficial in developing countries, fermentation offers both practical and economic benefits to an economy due to its low cost and ability to enrich the food substrate with flavour, aroma, texture and nutritional value (Starzynska-Janiszewska *et al.*, 2014). Fermentation can also improve the shelf-life of food, therefore preventing food loss due to spoilage in developing countries where canning, refrigeration and freezing is not accessible (Tamang *et al.*, 2016). The fermentation of legumes is an effective method to improve digestibility, nutritional content, and add value to crops.

2.3.3 Microorganisms used in fermentation

Microorganisms are the catalysts of fermentation. The microorganisms that show application in fermentation include bacteria, yeast and fungi. Their effectiveness during fermentation is highly-dependent on various factors, most importantly, the type of microorganism used to ferment the food. Several factors need to be closely-monitored to ensure that optimal microbial growth and activity occurs in a particular substrate. These include control over particle size, temperature, pH, time and moisture level/water activity. Without proper monitoring of these factors, the microorganism will not produce substantial amount of enzymes to drive the process

forward. The lack of enzyme production will result in a "stuck fermentation", with little improvement of digestibility and nutrition over time (Couto & Sanroman, 2006). There are three main groups of microorganisms that have been used in fermentation of legume pulses: lactic acid bacteria, *Bacillus spp*. and *Aspergillus spp*. These microorganisms have high productivity of enzymes which aid in the modification of the substrate. These three groups of organisms are ideal for food use due to their non-toxicity and Generally Recognized as Safe (GRAS) status (de Castro & Sato, 2014). Therefore, they are all excellent, viable choices when fermenting food.

2.3.4 Enzyme production

Fermentation enables microorganisms to produce enzymes which aid in the digestibility and functionality of the substrate. In most fermentation processes, amylase and protease enzymes are produced concurrently (Chutmanop *et al.*, 2008). Amylase functions in breaking down carbohydrates; whereas, protease is effective in hydrolyzing proteins by cleaving off peptides and freeing amino acids. These allow the microbes to assimilate nutrients for their metabolic activities more efficiently. Among these, proteolysis also results in a modified protein structure. Microbial hydrolysis of proteins impacts the structure by lowering the molecular weight in the peptide sequence, increasing ionizable groups and exposing hydrophobic groups. These changes can affect a protein's functionality-related properties, such as solubility, emulsification, foaming, water and oil holding capacity. The factors that affect the enzymatic hydrolysis of proteins are: the enzyme specificity, the extent of protein denaturation, the substrate used in the fermenting, enzyme concentrations, pH, ionic strength, temperature and absence or presence of inhibitory substances (Panyam & Kilara, 1996). Specificity of the enzyme is highly-important in order to modify the protein to improve digestibility and functionality. A hydrolyzing mechanism called endopeptidase is an enzyme that cleave peptides in the middle, thereby yielding two smaller peptides (Panyam & Kilara, 1996). This mechanism of endopeptidase provides more terminals for exopeptidase enzyme to act upon the protein. There are two types of exopeptidase enzymes which are distinguished based on their catalytic activity; aminopeptidases and carboxypeptidases. Aminopeptidases cleave amino acids from the amino terminal of the peptide sequence; whereas, carboxyl peptidases cleave the amino acid from the carboxyl terminal of the peptide sequence (Clemente, 2000). Endopeptidase mechanism generally produces larger peptides in comparison to exopeptidase. Clemente (2000) evaluated the enzymatic protein hydrolysates effect on human nutrition,

determining that the greatest degree of hydrolysis was the sequential reaction of endopeptidases and exopeptidase. Common problems associated with extensive protein hydrolysis is loss of functional properties and formation of bitterness. Bitterness occurs due to creation of lower molecular weight peptides containing hydrophobic amino acids (Panyam & Kilara, 1996). Therefore, it is important to monitor the degree of hydrolysis during fermentation to ensure that over-hydrolysis of the protein does not occur.

Enzymes produced also affect the nutritional potential of the substrate. The majority of therapeutic foods that are produced by the action of hydrolyzing mechanisms do so via exopeptidases which cleave smaller peptides (di- and tripeptides) from proteins making them easier for human absorption and digestibility in the body. Protein absorption as short-chain peptides (di- and tripeptides) is more efficient than that of free amino acids. The availability of short-chain peptides is greater due to peptide-specific transport systems and terminal phase of peptide digestion into amino acids via cytoplasmic peptidases within the enterocytes prior to circulation (Clemente, 2000). Therefore, fermentation and the production of enzymes can work to improve the absorption and digestion of proteins.

2.3.5 Bioactive peptides and antioxidants produced by fermentation

Fermentation is applied to improve bioactive components that influence health benefits and reduce anti-nutritional factors. During fermentation, microorganisms metabolically-breakdown complex organic compounds releasing several additional compounds known as bioactive peptides. Bioactive peptides are specific protein-derived fragments that have a positive impact on bodily functions and can influence human health (Kitts & Weiler, 2003). Within the sequence of the parent protein, bioactive peptides are inactive but become active once released during proteolytic activity. These bioactive peptides are then able to impact major body systems such as cardiovascular, digestive, nervous, gastrointestinal and immune system to reduce the risk of several lifestyle-related diseases. Many bioactive peptides have multifunctional properties, and therefore can have more than one impact. Sanjukta & Rai (2016) studied soybean fermented by different microorganisms to improve the bio-functional properties due to the increase in peptide content. The hydrolysis of soybean proteins by microbial proteases were found to produce antioxidant, ACE inhibitory, anti-microbial, anti-diabetic and anti-cancer properties. Therefore,

fermentation can be seen as a beneficial process due to its production of bioactive peptides that have the ability to improve human health.

Fermentation can also result in the reduction of anti-nutritional factors such as proteinase-inhibitors, phytic acid, lectins, tannins and oxalic acids. Anti-nutritional factors negatively impact the digestibility and bioavailability of nutrients, reducing the nutritional value of foods; therefore, the removal of toxic components will improve the value of these foods for human consumption. Reddy & Pierson (1994) evaluated natural or defined mixed-culture fermentations of plant foods and found that phytic acid content was reduced by up to 55% in tempe due to endogenous phytases produced by microorganisms during fermentation. Tannins, saponins and oxalates in some foods were also shown by Reddy & Pierson (1994) to be reduced by fermentation, although the mechanism was not determined. Additionally, fermentation eliminated lectins and enzyme-inhibitors, partially or completely, from legume foods.

2.3.6 Allergen attenuation via fermentation

Food allergens has become a growing problem in the food industry, and many studies have been performed to eliminate the allergenicity of soybean protein. Hydrolysis has been found to be an effective method to alleviate the allergenicity in legume proteins (Seo & Cho, 2016). Frias *et al.* (2008) studied the elimination of soybean products using SSF inoculated with *Aspergillus oryzae*, *Rhizopus oryzae* and *Bacillus subtilis*, as well as SmF inoculated with *Lactobacillus plantarum*. *Lactobacillus plantarum* showed the highest reduction in IgE immunoreactivity, followed by *B. subtilis* and *A. oryzae*. The authors indicated that the decrease in the allergenicity of soybean was due to the fermentation-based hydrolytic modification of peptides, altering the structure and thereby lowering the immunoreactivity. Their study concluded that fermentation could decrease soy immunoreactivity and potentially-hypoallergenic soy products could be made possible.

2.4 Effect of fermentation on protein functionality

Functional properties are physical and chemical properties that affect the nature of the protein in food systems (Boye *et al.*, 2010a). Protein is added to food not only for nutritional benefit but as well as for its functional properties, which ultimately contributes to the sensory quality of food. Multiple physicochemical properties of the protein interact to make up the complex and

desirable food system. In order for the food to be preferable, in many cases, multiple physicochemical properties of the protein are necessary (Hettiarachchy & Ziegler, 1994). Functional properties that are important to proteins to ensure the functionality in food products are solubility, emulsification, foaming, water and oil holding capacities. The functional properties play a fundamental role in the foods texture and organoleptic characteristics and are profoundly important in the production of products (Boye *et al.* 2010a).

2.4.1 Protein solubility

Protein solubility is an important property due to its influence upon other functional properties, such as foaming and emulsification. Protein solubility may be defined as the amount of nitrogen contained in a protein that dissolves in a solution under a specific set of conditions. Proteins may be soluble or insoluble in water, and typically dissolve in solution up to a maximum concentration. Highly-soluble proteins have greater dispensability of protein particles, which can then form colloidal systems. The application of the protein depends on the dispersion of the protein, and if it is highly-soluble then the potential for application is greater. Solubility is also a highly-important characteristic of liquid foods and beverages due to dispersion of particles, which influence texture and organoleptic characteristics (Adler-Nissen, 1976).

Protein solubility is dependent upon several factors such as hydrophobic and hydrophilic interactions, structure of the protein and environmental conditions. Hydrophobic interactions are protein-protein interactions; whereas, hydrophilic interactions are protein-solvent interactions. Hydrophobic interactions influence the protein's ability to self-aggregate and become insoluble in the solution. In contrast, hydrophilic interactions determine if the protein will stay in solution. Therefore, the ratio of hydrophilic and hydrophobic interactions is important in determining protein solubility and overall protein functionality (Wu *et al.*, 1998).

The protein structure (primary, secondary, tertiary and quaternary) is important to its solubility, and depending on the amino acid composition, molecular weight and native conformation determines how the protein will interact with the solvent. The structure solubility depends on the intermolecular and intramolecular interactions, and thus will accordingly precipitate out of solution or interact with the solvent (Pace *et al.*, 2004).

Environmental factors affecting protein solubility include pH, temperature, pressure, concentration of salts and protein concentration. The pH has an effect on protein solubility,

specifically whether the protein will remain dispersed in the solution or precipitate out of the solution. In order for a protein's solubility to be high, it needs to interact with the solvent. The pH affects protein solubility at the isoelectric point (pI) where the protein is insoluble. This is because the proteins have a net zero charge at the pI, therefore protein-protein interactions are stronger than the protein-solvent interaction. Many proteins are therefore insoluble, or minimally-soluble, at their pI. At a pH above the pI, proteins have a net-positive charge and at a pH below the pI, proteins have a net-negative charge causing greater protein-solvent interactions. At the pH below the pI proteins have the greatest solubility since more protein-solvent interactions are occurring.

Protein solubility is affected by temperature, as temperature increases solubility increase until it reaches the protein's denaturation point. At the point of denaturation, the protein is insoluble. The heat denatures the proteins, allowing them to unfold and expose buried hydrophobic groups, thereby disrupting the stabilizing hydrogen bonds. Hydrophobic interactions lead to aggregation, followed by precipitation, ultimately resulting in the loss of solubility (Carbonaro *et al.*, 1997). The effects caused by heating of proteins are irreversible since denaturation cannot be reversed.

Ionic strength affects protein solubility due to the charged ions competing for the solvent. At low ionic strengths, "salting-in" occurs whereby the ionic strength of the solution increases the solubility of the protein. Therefore, protein-solvent interactions are favored because the charge is lessened. At high ionic strength the opposite happens, where "salting-out" occurs. This results in insolubility of the proteins due to the proteins and ions competing for the solvent, where salt is strongly bound to the solvent and the protein molecules are unable to have strong interaction with the solvent (Zayas, 1997). In salt solutions protein solubility will increase (salting-in) until reaching a solubility maximum, at this point it will decrease (salting-out). Salting-out decreases the protein solubility by promoting protein-protein interactions. Salt reduces the thickness of the electric double layer that surrounds the individual proteins, thereby lessening its solubility. Salt also influences protein hydration, and therefore stability, depending on the nature of the salts present in the solvent. Salting-out causes the disruption of the hydration level of the protein and creates instability. Salting-in and -out are dependent upon the protein type, pH and temperature (Zayas, 1997).

Protein concentration can affect protein solubility, and once the maximum protein solubility is obtained the addition of more protein will lead to protein aggregation and precipitation.

Another major effect on the solubility of protein is processing conditions, which influences different functionalities of the protein. Processing conditions that effect protein solubility include pH during extraction, and precipitation or neutralization before drying of the protein. The degree of agitation or speed of blending will also affect protein solubility (Zayas, 1997).

Major proteins that pulses contain include globulins and albumins, which are salt-soluble and water-soluble, respectively. Boye *et al.* (2010b) found that the solubility of the majority of pulse proteins is highest at low acidic and high alkaline pH values. Solubility decreases near the pI, which occurs between pH 4 and pH 6 for pulses. Fermentation of pulses has been shown to improve the solubility in multiple cases. Prinyawiwatkul *et al.* (1997) studied the functional properties of fungal-fermented cowpea flour. The authors found an increase in solubility of heat-denatured proteins that was fermented with *Rhizopous microspores* subsp. *Oligosporus*. These results were attributed to the decrease in molecular size due to the degradation of the protein by the protease that was produced. In addition, more hydrophilic sites were exposed, increasing the overall solubility. Therefore, fermentation with fungal proteases can aid in increasing the solubility of protein.

2.4.2 Emulsifying properties of proteins

Emulsification is a fundamentally-important property in the manufacturing of foods due to its influence on appearance, flavour, rheological properties and stability. Emulsions are created by the mechanical agitation of two immiscible liquids; a discontinuous phase, which is dispersed as small droplets into a continuous phase. The formation and stability of emulsions is vital in food systems, such as salad dressing, mayonnaise and chocolate (Yu *et al.*, 2007). Food emulsions are generally two-phase systems; oil-in-water (O/W) emulsions or water-in-oil (W/O) emulsions. The difference between O/W and W/O emulsions is the texture that results; O/W systems are known to have a creamier texture, whereas W/O has a greasier texture (Zayas, 1997). Emulsifiers (e.g., sodium phosphate and monoglyceride) are commonly used in food processing to assist the formation and stabilization of emulsions. The emulsifying activity (EA) is the ability of the liquid to emulsify an oil to yield O/W emulsion. Whereas, emulsion stability (ES) is the ability of the emulsion to resist changes to its structure over a time period (Boye *et al.*, 2010b). ES is a critical factor in order to avoid separation of the food product into oil and liquid phases. It is the capacity of oil emulsion droplets to remain dispersed in liquid and not separate, resulting in creaming,

coalescing or flocculation of the product. Proteins are a suitable emulsifier to stabilize O/W emulsions due to their surface-active molecules, which work to lower the interfacial tension. Proteins have a greater hydrophilicity and a greater ability to disperse in the solution making them a suitable emulsifier. However, due to their larger droplet size, proteins do not reduce the interfacial tension as well as synthetic surfactants (e.g., lecithin and Tween) (Zayas, 1997).

In O/W solution, protein molecules generally migrate to the oil-water interface. Once at the interface, proteins re-align their hydrophilic groups towards the liquid and their hydrophobic groups towards the oil. This results in a change in protein configuration forming a looped-protein structure which prevents flocculation and coalescence of the oil droplets (Zayas, 1997). Modification of the proteins can influence their emulsifying activity; partial denaturation of protein offers improved emulsifying ability due to increased exposure of hydrophobic amino acids. Increased exposure of hydrophobic amino acids is important in enhancing emulsification ability due to their ability to interact with the oil droplets (Zayas, 1997). In addition, soluble proteins unfold due to the disruption of molecular forces, forming strong viscoelastic films surrounding the oil droplets. These protein films aid in stabilization of the emulsion.

The use of plant-based protein as an alternative emulsifier offers benefits such as affordability, abundance and wide-acceptance by consumers (Imbart *et al.*, 2016). Plant-based proteins have exceptional foaming and emulsifying agents, as they readily diffuse into newly-formed water-oil interfaces during the emulsification process. Commonly-used protein fractions with strong emulsifying properties currently on the market include soybeans, rapeseed, sunflower seeds and casein. Pea protein is potentially an excellent alternative to current marketed emulsifiers.

In order to demonstrate the potential for plant-based proteins to function as emulsifiers, various studies have been conducted. Udensi & Okoronkwo (2006), Amadou *et al.* (2010) and Xiao et al (2015) concluded in their studies that plant-based emulsifying properties could be increased through fermentation, due to proteolytic activity of the fermenting organisms. However, Imbart *et al.* (2016) reported that fermentation was not an effective processing method to increase the emulsifying properties of cowpea protein since denaturation of proteins lead to destabilization of the emulsion.

A study performed by Xiao *et al.* (2015) found fermentation with *Cordyceps militaris* SN-18 increased the emulsifying properties of chickpea flours. The authors attributed their findings to fungal proteolytic activity exposing hydrophobic groups, thereby altering the hydrophilic-

lipophilic balance to promote emulsification. During SSF of chickpea flour, larger protein molecules were broken down into smaller molecules. This aided in oil-water migration due to the smaller molecules and ultimately increased emulsifying properties. It was determined that shifting the pH away from the pI enhanced the emulsifying properties of chickpea flour.

Udensi & Okoronkwo (2006) studied the effects of natural fermentation on the functional properties of velvet bean (*Mucuna cochinchinensis)* protein isolate. The isolate was fermented for 72 hours and showed a significant increase in emulsifying capacity of the resultant protein isolates. This finding was attributed to partial hydrolysis of peptides to expose hydrophobic groups, which were then able to interact with the oil-water interface.

Amadou *et al.* (2010) studied the functional properties of soybean protein meal (SPM) processed by SSF with *Lactobacillus plantarum* Lp6. Three samples were evaluated unfermented SPM, fermented SPM and fermented SPM with added protease. The study showed unfermented SPM to have the highest emulsifying capacity (41.83 mL·0.5 g^{-1}), followed by fermented SPM with added protease during fermentation (38.09 mL·0.5 g^{-1}). Fermented soybean protein meal without the addition of protease obtained the lowest emulsifying value (28.01 mL·0.5 g^{-1}). The authors attributed these results to the addition of protease during fermentation, proteolytic hydrolysis of protein occurs resulting in oligopeptide content increasing and a decrease in polypeptide content. This study concluded that the addition of protease to fermentation sample or a high protease producing microorganism used in fermentation could increase the emulsifying capacity.

Imbart *et al.* (2016) studied the effects of germination and fermentation on the emulsifying properties of cowpea proteins. The study determined that fermented cowpea protein led to destabilization of the emulsion, which was caused by the degradation of proteins by microorganisms. The size distribution of the droplet broadened as the fermentation time increased due to the proteins being hydrolyzed during fermentation. Size distribution is important since the larger particles will be more likely to aggregate together and destabilize the emulsion. It was suggested that larger-sized droplets resulted in the unfolding of proteins around the droplets which decreased the physical interactions at the interface. In addition, viscosity was shown to decrease as fermentation time increased. Viscosity is an important characteristic of emulsifiers because the greater the viscosity, the greater the emulsification effectiveness. The viscosity effect is presumed to be due to oil droplets becoming physically restrained, thus preventing migration and droplets

coalescence. The study performed by Imbart *et al.* (2016) determined that the emulsion stability was weak in fermented cowpea protein and showed that after 48 hours the solution was clear at the bottom and cream formed at the top of the tube. The authors attributed the weak emulsion stability to the denaturation of proteins that occurred during fermentation, with unfolding of the proteins leading to a weaker interface allowing the droplets to coalescence.

2.4.3 Foaming stability and capacity of proteins

Foam is the entrapment of air in a liquid or solid to form small bubbles that impart texture in food products. The ability of proteins to form stable foams is important in a multitude of food applications including cakes, beverages, breads and whipped toppings (Chandra-Hioe *et al.*, 2016). The air cells impart smoothness and lightness to the food product, uniform rheological properties and enable dispersion of flavours (Kinsella, 1981). Therefore, foams are very important for taste, texture and providing cheap functionality with exceptional stability (Green *et al.*, 2013). Foods where foaming is important are two-phase systems, where the dispersed phase is made up of air cells separated by a thin continuous liquid layer called the lamellar phase (Zayas, 1997). Foams can be gas in liquid (G/L) dispersions (e.g., meringue) or gas in solid (G/S) dispersions (e.g., breads and cake). Depending on the continuous phase, foams are formed by the entrapment of air which becomes surrounded by protein films, allowing G/L or G/S dispersions to form stable phases. These systems are generally unstable and the need of a surfactant molecule to orientate at the air-water interface is necessary (Makri *et al.*, 2005). Proteins can act as surfactant molecules and different proteins have different abilities to form and stabilize foams in food systems.

The most commonly-used foaming agents from protein sources are egg whites, gelatins, casein, gluten, soy proteins and whey protein (Barac *et al.*, 2015). However, these foaming agents may not be suitable for all dietary needs since they are associated with allergenic properties. Thus, pea protein would be a good alternative on the market as a foaming agent due to its low cost, wide availability and limited allergen association. Foaming properties of proteins are identified by their foaming capacity and foaming stability. Foaming capacity (FC) is the maximum volume increase due to dispersed proteins (i.e., how much air can be trapped within the unit amount of protein). Foaming stability (FS) is the ability of foam to remain the same over an allotted time period. These properties are independent from one another; e.g., the proteins can function well as foaming agents

but have poor stability. Instability of foams is influenced by the drainage of liquid from the lamellae and increased size of bubbles followed by rupture (Sathe *et al.*, 1982).

Foam formation depends on the protein's ability to rapidly diffuse and the adsorption of various proteins at the air-water interface (Martin *et al.*, 2002). The stability of the foam is dependent on the protein's ability to lower the interfacial tension. Similar to emulsions, the interfacial tension plays an important role in the stability of the foam. High interfacial tension leads to destabilization of the foam due to the coalescence of bubbles. Therefore, lowering the interfacial tension by the addition of proteins will stabilize the foam and increase the FC. The basic application of creating a good foam is to decrease the interfacial tension. In order to achieve the optimum FC, proteins with low molecular weight, high surface hydrophobicity, high solubility, greater flexibility and ease of denaturation are favorable. Lower protein molecular weight is favorable for increased foaming due to their greater ability of protein absorption at interface. High surface hydrophobicity of proteins enhances air-to-air interactions and increases the speed of molecular orientation at the interface. An increased solubility increases the surface activity of proteins which increases the foaming ability due to the decreasing of protein molecular weight and exposure of hydrophobic amino acids to the solvent (Murray, 2007). Flexible proteins have the ability to align at the interface more rapidly and thereby reduce the surface tension. Denaturation of the protein also aids in its alignment at interface, e.g., globular proteins cannot surface-denature, resulting in low foaming ability. The majority of proteins found in legumes are globular in nature; therefore, legumes have lower foaming properties in comparison to animal-based proteins (Kaur & Singh, 2007). Foams are stabilized the best when electrostatic repulsion is minimized. Electrostatic repulsion can be minimized at the protein's pI, or by salt addition to screen charges. Proteins are better foaming agents than small molecular weight surfactants because they can lower the interfacial tension as well as form a continuous and highly-viscous film at interface by intermolecular interactions (Makri *et al.*, 2005).

Knowledge regarding the foam formation, capacity and stability characteristics of proteins is necessary for the incorporation of protein ingredients into foods. Plant-based proteins are novel in that they have potential of being good foaming agents in food systems. Udensi & Okoronkwo (2006) and Yu *et al.* (2007) studied the physicochemical functions of fermented legume varieties, where they both determined that fermentation increased the FC. This was due to the amphipathic properties of the protein enabling lower interfacial tension. However, Obatolu *et al.* (2007) and

Chandra-Hioe *et al.* (2016) found that fermentation decreased the foaming capacity and stability of plant protein in comparison to the unfermented samples. The authors attributed the decrease in foaming properties on the type of legumes fermented, in addition to the amount of denaturation that occurred.

Udensi & Okoronkwo (2006) studied natural fermentations impact on functional properties of velvet bean (*Mucuna cochinchinensi)* protein isolate. The results indicated that fermentation significantly increased the FC and FS. Specifically, the foams produced were found to be stable after a two-hour period. These results suggested fermented velvet bean isolates use in foam-based foods could alleviate nutritional problems in developing countries. Yu *et al.* (2007) studied the effect on functional properties of peanut protein concentrate when processed by two different methods: roasting and fermentation, in addition to a combination of roasting followed by fermentation. The fermentation was carried out using three varieties of peanut flour (i.e., defatted, defatted roasted, partially-defatted and fermented) inoculated with *R. oligsporus*. The foaming functionality of the defatted peanut flour was found to be an ineffective foaming agent. Roasting reduced the FC by half; however, the fermentation of roasted peanut flour increased the FC by 3-fold. The authors attributed this to amphipathic characteristics of protein making them effective foaming agents that work at air-water interface ultimately preventing destabilization of the foam. Chandra-Hioe *et al.* (2016) studied the functional properties of fermented chickpea and faba bean flour. The flours were fermented using a yogurt culture containing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus* sp.. They found the FC and FS were significantly lower in the fermented flour samples than raw flour samples ($p<0.05$). The FC and FS varied on the type of legume fermented: kabuli chickpea had the greatest FS and the lowest FC. Whereas, faba bean had the greatest FC and the lowest FS. This could be attributed to the composition of the amino acids making up the legume protein, as well as other compounds found in the protein (i.e., allowing a higher FC or FS). For example, both non-protein nitrogen compounds and carbohydrates are presumed to stabilize foams (Obatolu *et al.*, 2007). Obatolu *et al.* (2007) studied the processing and functional properties of yam bean flour fermented using lactic acid bacteria. The FC was found to decrease when fermented (7.8%) in comparison to the raw flour FC (40.2%). When comparing fermentation to the other processes (boiling and roasting), the fermented flour was significantly higher in FC. The results were attributed to the flour samples being denatured which significantly decreased the FC. Boiled and roasted flours experienced a more destructive heat treatment in

comparison to the fermented flour. Therefore, when processing legume flours for FC, fermentation is considered the most viable choice due to limited denaturation of the protein (Obatolu *et al.*, 2007).

2.4.4 Oil holding capacity of proteins

Oil holding capacity (OHC) is the ability of a matrix of proteins to physically entrap and hold an amount of fat. OHC is an essential characteristic with potential to enhance mouth-feel, texture and flavour of the food product (Yu *et al.*, 2007). From the food industry's point of view, it is important to have OHC since it reflects the emulsifying capacity, which is necessary to attain the correct rheology and avoid separation of the product. High OHC is necessary in a variety of foods, such as ground meal formulation, meat replacers and extenders, doughnuts, baked goods and soups (Kaur & Singh, 2007). The capacity of oil holding is influenced by the protein's matrix structure, the type of fat and the fat distribution. Protein's matrix structure is important in determining how much oil the protein can retain, and this depends upon protein strand size and pore size; the greater the porosity, the greater the entrapment of fat (Ma *et al.*, 2011). Smaller droplets are able to entrap and absorb a higher amount of oil; whereas, larger oil droplets are unable to hold as much oil due to their low surface area. The oil distribution can be influenced if there is an emulsifying agent included or excluded. An emulsifying agent will allow better distribution and greater oil holding in comparison to the exclusion of the emulsifying agent (Hall, 1996).

Lipid-protein interactions are important in determining the amount of oil holding that can be retained. Non-polar side chains of protein molecules are the primary location of lipid-protein interactions; therefore, the number of available non-polar sites are critical. The greater amount of non-polar side chains available to interact with lipids, the greater of OHC the product has. Hydrophobicity also plays a role in lipid-protein interactions, with more hydrophobic and insoluble proteins being better able to interact with lipids and bind them together resulting in a higher OHC. The interactions between proteins and lipids are important for the formation of specific functional properties in foods, such as fat emulsification in meats, fat entrapment in sausage batters, flavour absorption and dough preparation (Zayas, 1997).

There have been numerous studies attempting to increase the OHC of legume flours through fungal fermentation, although inconsistent results have concluded. Prinyawiwatkul *et al.* (1997); Yu *et al.* (2007) and Xiao *et al.* (2015) found that fungal fermentation increased OHC due

to increased exposure of hydrophobic interior proteins. In contrast, Obatolu *et al.* (2007) found that OHC decreased after fermentation due to the lack of protein denaturation and overall insufficient exposure of non-polar groups in which lipid-protein interactions could occur. It was concluded that other processing methods, such as boiling, would be a more viable method to increase the OHC in certain cases.

Yu *et al.* (2007) found raw peanut flour that was fermented with *R. oligsporus* marginally-decreased fat retention of the product from 2.7 g/g to 2.3 g/g, although a significant enhancement was also found in roasted peanut flour from 1.7 g/g to 2.5 g/g. These results could be the consequence of denaturation during roasting allowing the exposure of hydrophobic sites, and therefore exposing available non-polar sites that allow lipid-protein interactions to occur. It was concluded from the Yu *et al.* (2007) study that fungal fermentation significantly increased all functional properties of raw and roasted peanut flours. Additionally, Prinyawiwatkul *et al.* (1997) found similar results upon determining the effect of fungal fermentation inoculated by *R.* microspores subsp. *oligosporus* as well as, soaking and boiling effect on the functional properties of cowpea flour. It was found that the fermentation and boiling only increased the OHC slightly, ranging from 0.7 g/g to 0.9 g/g. Boiling was concluded to have the greatest increase in functionality on cowpea flour due to the ability to expose non-polar groups (Prinyawiwatkul *et al.,* 1997). A study performed by Xiao *et al.* (2015) determined the outcome of functional properties of chickpea modification with SSF using *Cordyceps militaris* SN-18. The OHC of chickpea flours were shown to increase 18.9% as a consequence of *C. militaris* fermentation and was attributed to fermentation increasing the availability of hydrophobic amino acids by unmasking the non-polar residues from the interior protein molecules. Therefore, allowing more lipid-protein binding to occur (Xiao *et al.*, 2015). Obatolu *et al.* (2007) studied the functional properties of five flours from yam beans, one of which was fermented using lactic acid bacteria. In this study, it was found that fermentation significantly lowered the OHC (from 0.6 g/g to 0.4 g/g) of the raw variety. Furthermore, the results were also lower in comparison to other processing methods, such as boiling, roasting and malting. The increase in oil absorption is correlated with heat dissociation and denaturation of the protein, enabling the interior protein molecules and non-polar residues to become exposed. The study concluded that the processing method has a great influence on the outcome of OHC, where the increase in OHC can be attributed to the effect of heat treatment.

2.4.5 Water holding capacity of proteins

Water holding capacity (WHC) is the ability to hold water within the protein matrix and retain the water against gravitational, mechanical and thermal forces. The WHC is important for food processing applications, storage of the product and plays a major role in the texture and flavour of the food. Protein WHC correlates with water binding capacity, which is the grams of water bound per gram of protein (Boye *et al.*, 2010b). Water binding is determined by the protein's composition and conformation; therefore, water binding capability can be estimated by evaluating the protein's amino acid composition (Zayas, 1997). Water retention is the water absorbed or retained by the food's constituents, and also influences texture as well as colour and sensory properties. Functional properties are dependent on the protein-water interaction and hydrogen bonds. These interactions effect functional properties such as water binding and retention, solubility, emulsifying properties, viscosity, and gelation (Zayas, 1997).

Water is held in a protein structure and can be categorized as absorbed water and retained water. Absorbed water surrounds the protein molecule with water tightly-bound to specific, charged sites. Absorbed water is made up of two types of water, vicinal water and multi-layer water. Vicinal water is the first layer of water surrounding the protein, the water molecules are held tightly to the protein surface by hydrogen bonds. Multi-layer water interacts with water-water and water-protein hydrogen bonds. It is the second layer surrounding the protein that is made up of numerous layers and covers the remaining exposed sites on the protein. Retained water is trapped in the protein matrix and is made up of bulk water that can be free or entrapped within the protein matrix. The amount of water entrapped is influenced by the matrix's pore size (Zayas, 1997).

Absorbed water is highly-dependent on the intrinsic properties of the protein, such as amino acid composition, size, protein structure, pI, protein concentration and the number of exposed polar groups. Amino acid composition of the protein affects how much water can be bound and retained. If the amino acid profile is hydrophobic it will result in a lower WHC; whereas, if the amino acid profile is hydrophilic the protein will have a higher WHC capacity since the peptide bonds will interact with water, thereby forming bonds. WHC is affected by protein size; the larger the protein, the greater the water holding potential due to the relatively larger surface area to volume ratio (Shirota *et al.*, 2008). The structure of the protein influences the WHC of the protein. The nature of the structure, e.g., either globular or fibrous, dictates the number of water-

binding sites available. Globular protein structure is loosely-bound to water molecules due to its folded peptide chain orientation, and therefore, it is able to hold less water than fibrous-structured proteins. Fibrous protein structure is able to bind with water molecules tightly and therefore results in a higher WHC.

The pI is another important determinant of WHC, and pI is affected by the pH and surface polarity. The pH is an extrinsic property that relates to pI. The further away the pH is from the protein's pI, the greater its water-binding tendency, due to its greater charge. The converse situation also holds; the closer the pH is to the protein's pI, the more difficult it will be for the water to bind to the protein, due to the reduced charge. The pH can alter the WHC through net charge effects, with the pI being the condition where the protein's net charge is zero. At pH values below the pI, the net charge is positive, and a net negative charge occurs at pH values above the pI. The size of the distance the pH is from the pI influences the amount of charged groups which are able to bind with water (Hui, 2005).

Protein concentration can affect WHC since a higher concentration of protein would yield more protein-protein interactions, leaving fewer exposed groups for protein-water interactions. In comparison, a lower protein concentration would have a higher WHC since there would more exposed groups with the ability to form protein-water interactions. A higher number of exposed polar groups allows for greater WHC, since polar groups bind with water to form hydrogen bonds (Zayas, 1997).

Extrinsic properties also influence a protein's WHC, and include pH, salt, temperature, pressure and processing conditions. Salt affects WHC and high salt concentrations in food systems can cause screening of the charge, causing proteins to aggregate together and decreasing their WHC. High temperature further affects WHC by breaking hydrogen bonds; thus, lower temperatures are better for protein-water hydrogen bonds and overall higher WHC values (Hui, 2005).

There have been various studies on the fermentation of legumes inoculated with fungi or bacteria to evaluate potential benefits of fermentation to a food's WHC. It was found by Obatolu *et al.* (2007),Yu *et al.* (2007), and Xiao *et al.* (2015) reported that fermentation increased the WHC due to the exposure of polar amino acid groups and ability to bind more water. However, Adebowale & Maliki (2011) found that WHC decreased over a period of time due to the inability of the fermentation process to expose polar groups on the surface of the protein to allow water

binding. The impact of fermentation on WHC is important when incorporating protein into products, otherwise products could have incorrect texture with short shelf-lives (Boye *et al.*, 2010a).

Food processing methods, such as fermentation, affects intrinsic factors by altering the protein conformation and in turn the hydrophobicity of the protein. Yu *et al.* (2007) found that the WHC of fermented peanut flour inoculated with *Rhizopus oligosporus* increased in comparison to the raw peanut flour. The increase in WHC was attributed to the higher protein solubility, which was due to the proteolytic activity of the fungal enzymes. The fungal enzymes cleaved peptides forming soluble oligopeptides and amplified the amount of water binding sites via proteolysis.

Obatolu *et al.* (2007) examined the effect of various processing methods (boiled, roasted, malted and fermented) on the functional properties of yam bean flour. In this study, yam bean flour was inoculated with lactic acid bacteria to ferment. It was determined when measuring the WHC after 48 hours of fermentation that it increased slightly in comparison to the raw unprocessed yam bean flour. This was attributed to fermentation denaturing the protein, revealing polar amino acids and allowing more water to bind to the protein.

Xiao *et al.* (2015) demonstrated that the WHC increased during SSF with *Cordyceps militaris* SN-18 on chickpea flour. In this study, the WHC was evaluated over a pH range (4.0, 5.0, 6.0 and 9.0), where no significance of pH on the WHC revealed. However, fermentation significantly increased the WHC of chickpea flour at all the pH values studied. The authors attributed the increase in WHC of chickpea flour to the production of proteolytic activity during fermentation which resulted in the breaking of peptide bonds in the protein. Thus, increasing the number of polar groups and in turn increasing the hydrophilicity of the proteins.

Adebowale & Maliki (2011) studied the effect of fermentation on the functional properties of pigeon pea seed flour, where it was found that WHC decreased after fermentation. It was observed that the WHC decreased as the fermentation time increased; on day 1, the WHC was 142.0 g/100 g; whereas, on day 5, the WHC was 113.0 g/100 g. However, the WHC was greater compared to other values obtained from other legumes such as soybean, *Mucuna*, and lupin seed flour. Thus, WHC also depends on legume type, and varies from species to species due to the protein composition. The authors found that pigeon pea had a greater WHC in the incorporation of bakery products. The study concluded that limited fermentation generally-improved WHC of

pigeon pea. This indicated that length of time for fermentation is an important factor to achieve the best WHC.

2.5 Fermentation effect on protein bioavailability and quality

Proteins are nitrogen-containing macromolecules formed by a linear chain of amino acids (a polypeptide) linked by covalent peptide bonds. Amino acid components vary among the source, especially animals and plants. Food protein from animal sources provide a complete source of protein, whereas plant-source proteins lack one or more of the essential amino acids (Hoffman & Falvo, 2004). Essential amino acids cannot be synthesized by the human body and therefore are vital to consume in our diets. The absence of any essential amino acids will compromise the ability of tissue to grow, be repaired or maintained (Hoffman & Falvo, 2004). Protein quality is defined as the ability of a food protein to meet the body's metabolic action and is determined by its amino acid composition, digestibility and bioavailability (Millward *et al.,* 2008). Quality encompasses the availability of amino acids that it supplies, and digestibility refers to how the protein is best utilized (Hoffman & Falvo, 2004). Bioavailability may be defined as the proportion of ingested dietary amino acids that are absorbed in a chemical form suitable for utilization for protein synthesis in our body. There are many factors that affect the protein digestibility and bioavailability, such as the amino acid profile, protein conformation, the make-up of the food matrix, biological differences of individuals and food processing. Deficiency of micronutrients (e.g., vitamin A, iron, iodine and zinc) is a common problem in developing countries due to food staples containing anti-nutritional factors that affect the bioavailability. Heating during processing is an effective method to increase the absorption of micronutrients, such as iron, due to the softening of the food matrix and the release of protein-bound iron (Lombardi-Boccia *et al.,* 1995). Hemalatha *et al.* (2007) studied the influence of fermentation on grain and legume flour in attempt to increase zinc and iron bioavailability in the final product. Fermentation of cereals and legumes was found to be an effective method to reduce inhibitors of mineral absorption, in particular phytic acid and tannin, therefore enhancing zinc and iron absorption.

The methods of measuring protein quality currently do not account for bioactive compounds in protein that inhibit digestibility (Millward *et al.,* 2008). Thus, quantity and quality of proteins are not in the linear relations, and the components, structure, physicochemical nature can affect the quality of proteins as nutrients. Furthermore, the protein quality must be evaluated

in a separate way. The assessment of protein quality can be measured in a number of ways; protein efficiency ratio (PER), biological value, net protein utilization, protein digestibility corrected amino acid score (PDCAAS) and digestible indispensable amino acid score (DIAAS), that can be determined by i*n vitro* or *in vivo* methods.

The two best known and widely-utilized methods are PDCAAS and DIAAS. PDCAAS method is based on the determination of protein content, amino acid profile and protein digestibility using true fecal digestibility of the entire protein. Whereas, DIAAS measure amino acids by evaluating each individual amino acid as a constituent of food (Marinangeli & House, 2017). PDCAAS is a simple procedure that is an internationally-approved method for protein quality assessment. PDCAAS measures protein based on consumer target age and amino acid reference which are then used to estimate the digestibility of the protein. The proteins cannot attain a score higher than 100% when compared to the reference score. Therefore, complimentary protein sources such as cereals and pulses are not able to attain a score higher than 100% even though their quality is much higher than proteins that lack an essential amino acid (Hoffman & Falvo, 2004). PDCAAS has some major disadvantages, including the inability to credit the extra nutritional value of proteins when they attain a higher score than the reference protein, the failure to account for the presence of anti-nutritional factors, the overestimation of protein value in the elderly, and the failure to account for the influence of the ileal digestibility (Sarwar, 1997). The DIAAS method measures dietary protein quality based on true ileal amino acid digestibility determined for each amino acid individually. The Food and Agriculture Organization of the United Nations (FAO) proposed that PDCAAS is to be replaced by DIAAS in the near future, due to the fact that this method gives a better estimation of absorbed amino acids since it is measured at the distal end of the small intestine (Rutherfurd *et al.*, 2015).

Fermentation could potentially be a successful process to improve the overall protein quality of legumes. Cuevas-Rodriguez *et al.* (2004) studied maize tempeh flour through fungal solid-state fermentation using the *in vitro* protein digestibility to determine the protein quality. It was determined that the SSF process significantly improved the nutritional value, the true protein content and increased the *in vitro* protein digestibility increase. The increases in *in vitro* protein digestibility are attributed to the reduction of anti-nutritional factors, protein denaturation during cooking and protein hydrolysis during fermentation, resulting in proteins that are more susceptible to enzyme action.

3. MATERIALS AND METHODS

3.1 Materials

Pea protein concentrate was kindly donated by Parrheim Foods in 2016 (Saskatoon, SK, Canada) for this research. Fungal strains (*Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590) were obtained from Agriculture Research Service, US Department of Agriculture (Peoria, IL, United States). All chemicals used in this study were purchased from Sigma-Aldrich Co. (Oakville, ON, Canada) and were of reagent grade.

3.2 Methods

3.2.1 Fermentation

(a) Microorganism and culture conditions: Fungal strains (*Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590) were cultivated on Potato Dextrose Agar (PDA) and incubated at 30ºC for 7 d under aerobic conditions. In an aseptic environment, a colony was transferred from a previous cultivated microorganism petri dish using a loop and placed into 8 mL of peptone water. The sample was vortexed for approximately 30 s to 1 min to suspend spores in the peptone water. Once suspended, 100 µL was pipetted onto the PDA and using a spreader to spread the spores over the agar. After 7 d, the plates were maintained at 4ºC and sub-cultured every 2 months. Fungi selected for this study have been designated as generally recognized as safe (GRAS) and thus may be used for human and animal food applications (Jung *et al.*, 2005).

(b) Spore suspension preparation: Spores for use in fermentation studies were grown on PDA agar plates. Spores from a single agar plate were suspended in 10 mL of deionized water, and the concentration of spores were determined by direct microscopy counting (Leica, Model S6E, Wetzlar, Germany) using a hemocytometer (Bright-Line, Horsham, PA, USA). Both strains were standardized to a spore concentration of 10^7 colony forming units (CFU) per gram of substrate prior to use in fermentation tests.

(c) Solid-state fermentation: PPEF (230 g) was inoculated with a 1 mL aliquot of the spore suspension per gram of substrate (10^7 spore/g substrate) at 50% moisture. The moisture content

was calculated using Eq. 1, below, to determine the amount of water to add in addition to the spores:

$$M_n = \left(\frac{W_w - W_d}{W_w}\right) \times 100 \quad \text{(Eq. 1)}$$

where, M_n is the moisture content (%) of material of n, W_w is the wet weight of the sample, and W_d is the weight of the sample after drying. Using a KitchenAid mixer, the sample was mixed at high speed for approximately 3 min before being spread out thinly (approximately 25 mm) and evenly onto a sheet pan. Inoculated batches were incubated at 30 and 40ºC in an Isotemp incubator (Fisher Scientific, Model 650D, Waltham, MA, USA) and allowed to ferment for 6 h. Samples were taken at the time of initial inoculation (T = 0), and thereafter at 2, 4 and 6 h for pH, degree of hydrolysis, functionality and proximate measurements. Fermentation tests were repeated 3 times, using separate spore suspensions for each batch to make triplicate batches. Samples (80 g) of fermented PPEF were removed from each batch at defined intervals (see above), deactivated at 80°C in a water bath and suspended in deionized water adjusted to pH 8.0 in order to neutralize the pH of the sample. Samples were then frozen at –20°C, and freeze-dried for 48 h into a powder using a freeze drier (Labconco, Freezone 12, Kansas City, USA). The dried powder was stored at room temperature (21°C-23°C) for further testing.

3.2.2 pH determination

The pH was determined according to Adinarayana *et al.* (2004). A 1-g portion of fermented PPEF was removed at 0, 2, 4 and 6 h and suspended in 10 mL of distilled water. The solution was mixed for 20 min and then measured using a pH meter. Measurements were performed in duplicate, for each of the triplicate batches (n = 3), and reported as the mean ± standard deviation.

3.2.3 Determination of degree of hydrolysis

One gram of fermented PPEF (at each time point and temperature) was removed and added to 20 mL of sodium phosphate buffer at pH 8.2 (w/v) within 50 mL centrifuge tubes. The tubes were capped and then placed in a hot water bath (VWR Scientific Products, Radnor, PA, USA) at 95ºC for 2 min to inactivate all vegetative microorganism and active enzymes. Samples then removed from water bath and left on the lab bench top until cooled to room temperature (20-23ºC). Samples were centrifuged at 8,228 × g for 30 min using a 5804R centrifuge (Eppendorf, Hamburg,

Germany). The supernatant was removed and further analyzed following a method by Adler-Nissen (1979). This method assesses the colour change of the reaction between protein and Picrylsulfonic acid to yield N-trinitrophenyl-protein derivatives. Samples were poured into plastic cuvettes and measured using a Genesys 10S UV-VIS spectrophometer (Thermo Scientific, Madison, WI, USA) at 340 nm to determine the molecular absorption. Measurements were made in duplicate, on each of the triplicate batches (n = 3), and reported as the mean ± standard deviation. A 1.5 mM glycine solution was used to create a standard curve.

The total acid hydrolysis was also measured as part of the degree of hydrolysis calculation according to the methods of Adler-Nissen (1979) and Jung *et al.* (2005). The total acid hydrolysis was determined by adding 24 mg of PPEF to a screw cap Pyrex tube with 15 mL of 6.0 N HCl. The tubes were then purged with O_2-free nitrogen gas, and incubated in a forced air oven at 110ºC for 20 h. After 20 h, the tubes were removed and adjusted to pH 7.0 with 2 M NaOH. The samples were filtered through Whatman Grade 3 filter paper, and then 250 µL aliquots of sample were added to 2.00 mL of 1% SDS solution in buffer. This was followed by the addition of 250 µL to 2.00 mL of 5 mM buffer. The sample blank was prepared by adjusting a solution of 6.0 N NaOH and 6.0 N HCl to obtain a pH of 7.0. This was performed in triplicate and analyzed by the previous method above. Total acid hydrolysis and degree of hydrolysis were calculated using formulas (Eqs. 2 and 3) below:

$$h = (h_t - h_c)' \, DF \qquad \text{(Eq. 2)}$$

$$\%DH = \left(\frac{h}{h_{tot}}\right)' 100 \qquad \text{(Eq. 3)}$$

where h is the yield of hydrolysis equivalents of α-NH_2-glycine equivalents, h_t is the mM concentration of α-NH_2-glycine equivalents, h_c is the mM concentration of α-NH_2-glycine equivalent taken at time 0 before microorganism is added and fermented and DF is the dilution factor. The data was presented the mean ± one standard deviation derived from PPEF derived from the triplicate fermentation batches (n=3).

3.2.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

To determine the extent of protein hydrolysis, and changes to the size of protein an SDS-PAGE was performed using the method of Laemmli (1970) using a 12% separating gel at pH 8.6

ad 4% stacking gel at pH 6.8. Protein samples, 1% (w/w), were left to stir overnight and the following morning, 50 µL of solution was then dispersed in 50 µL of 2× SDS-PAGE sample buffer containing 20 mM Tris-HCl buffer at pH 7.6, 10% SDS solution, 2% β-mercaptoethanol, 50% (v/v) glycerol and 0.01% bromophenol blue, and then heated for 10 min at 85ºC, followed by centrifugation at 7,500 × g for 10 min. Subsequently, the gel was stained with 0.25% Coomassie blue stain for 1 h, followed by de-staining with de-ionized water overnight. The protein bands were then imaged, where images are used to estimate the molecular weight determination against a set of standards. Protein bands were quantified using ImageJ® (National Institutes of Health Bethesda, Maryland, USA). The protein bands were measured via volume, where volume is determined by the sum of pixel intensity for all pixels in each section.

3.2.5 Physicochemical properties

(a) Proximate composition. The proximate composition of fermented PPEF were carried out according to the Association of Official Analytical Chemists (AOAC) methods 923.03 (crude ash), 920.87 (crude lipid), 984.13A (crude protein; %N × 6.25), and 925.10 (moisture) (AOAC, 2005). Crude protein, ash and lipid values were reported on a dry weight basis (d.b.). Measurements were made in triplicate, on composited samples (n = 3), and reported as the mean ± standard deviation.

(b) Surface charge. Fermented PPEF powder was dispersed in deionized water at a concentration of 0.05% (w/w) pH adjusted to 3.0, 5.0 or 7.0 using 0.1 M HCl or NaOH, and allowed to stir overnight (16 h) using a mechanical stirrer. The surface charge (or zeta potential) of each sample was determined according to Can Karaca *et al.* (2011) by measuring the electrophoretic mobility of fermented PPEF samples using a Zetasizer Nano (Malvern Instruments, Westborough, MA, USA). The zeta-potential (ζ; units: mV) was determined from the electrophoretic mobility (U_E) using Henry's equation (Eq. 4), as follows:

$$U_E = 2\varepsilon \cdot \zeta \cdot f(\kappa\alpha)/3\eta \qquad \text{(Eq. 4)}$$

where η is the dispersion viscosity, ε is the permittivity, and $f(\kappa\alpha)$ is a function related to the ratio of particle radius (α) and the Debye length (κ). Measurements were made in triplicate, on the composite batch (n = 3), and reported as the mean ± standard deviation.

(c) Surface hydrophobicity. Fermented PPEF powders were dispersed in deionized water at a concentration of 0.025% (w/w), pH adjusted to 3.0, 5.0 or 7.0 using 0.1 M HCl or NaOH, and allowed to stir overnight (16 h) using a mechanical stirrer prior to measurements. Surface hydrophobicity of fermented samples was determined using the fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS), and the modified method of Kato and Nakai (1980). The pH-adjusted stock solution was then diluted to obtain final protein concentrations of 0.005, 0.010, 0.015, and 0.020% (w/w). Then, 20 µL of an 8 mM ANS solution (in deionized water at desired pH 3.0, 5.0 or 7.0) was added to 1.6 mL of each protein concentration and vortexed for 10 s and kept in the dark for 5 min. The fluorescence intensity was measured using a FluoroMax-4 spectrophotometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) with excitation and emission wavelengths of 390 and 470 nm, respectively, and a slit width of 1 nm. Sample blanks was prepared by adding 20 µL of deionized water (pH 3.0, 5.0 and 7.0) instead of the ANS probe. The initial slope of a plot of the fluorescence intensity [protein solution with probe minus the same protein solution without the probe] versus protein concentration was calculated using linear regression analysis and used as an index of surface hydrophobicity. All intensity data was arbitrarily divided by 10,000 prior to statistical analysis and graphing. Measurements were made in triplicate, on the composite batch sample (n = 3), and reported as the mean ± standard deviation.

3.2.6 Functionality

(a) Nitrogen solubility. In brief, a 1% (w/w) solution of fermented PPEF was prepared by dispersing the powder in deionized water, pH-adjusted to 3.0, 5.0 or 7.0 using 0.1 M HCl or NaOH, and allowing it to stir overnight (16 h) at room temperature (21°C-23°C). The suspension was then centrifuged at 3,070 × *g* for 10 min using a centrifuge (Eppendorf, Model 5804R, Mississauga, ON, Canada). The percent nitrogen was determined as a ratio of nitrogen measured in the suspension to the original amount in fermented samples used. Nitrogen levels within the fermented PPEF and the supernatant after extraction was determined using the AOAC Method 920.87 (AOAC, 2005). Measurements were made in duplicate, on each of the triplicate batches (n = 3), and reported as the mean ± standard deviation.

(b) Emulsifying properties. The emulsifying activity (EA) and emulsion stability (ES) of fermented PPEF were performed according to Kaur and Singh (2005). A 1-g portion of PPEF was dispersed in 14.2 mL of deionized water, pH adjusted to 3.0, 5.0 or 7.0 using 0.1 M HCl or NaOH

and allowed to stir using a mechanical stirrer overnight (16 h) at room temperature (21°C-23°C). The solution was then homogenized using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) equipped with a 20-mm saw tooth probe at speed 4 (~10,000 rpm) for 30 s. Then 7.1 mL of canola oil was added to the solution and homogenized for 30 s. Next, 7.1 mL of canola oil was added to the solution and homogenized for 90 s. After homogenization, the emulsion was centrifuged at 1,300 × g for 5 min using a centrifuge (Eppendorf, Model 5810R, Mississauga, ON, Canada). The EA represents the height of the emulsion and was measured using a micrometer. The EA was then determined using Eq. 5, below:

$$Emulsion\ activity\ (\%) = \frac{Height\ of\ emulsion\ layer}{Height\ of\ whole\ layer} \times 100 \quad \text{(Eq.5)}$$

The ES is the ability of the emulsion to resist changes to its structure over a time period. The ES was measured using the prepared emulsion. The emulsion was heated in a water bath (VWR Scientific Products, Radnor, PA, USA) at 85ºC for 30 min. The emulsion was then removed from the water bath and placed in a second water bath at 23ºC for 15 min. The emulsion was then centrifuged at 1,300 × g for 5 min using a centrifuge (Eppendorf, Model 5810R, Mississauga, ON, Canada). The ES was determined using Eq. 6, as follows:

$$Emulsion\ stability\ (\%) = \frac{EA\ after\ heating}{EA\ (unheated)} \times 100 \quad \text{(Eq. 6)}$$

Measurements were made in duplicate, on each of the triplicate batches (n = 3), and reported as the mean ± standard deviation.

(c) Foaming capacity. The foaming capacity (FC) was determined according to Liu *et al.* (2010). A 1% (w/w) solution of fermented PPEF was prepared by dispersing the powder in deionized water, pH adjusted to 3.0, 5.0 or 7.0 using 0.1 M HCl or NaOH, and stirring (~250 rpm) overnight (16 h) at room temperature (21°C-23°C). A 15-mL aliquot was then transferred to a 400 mL beaker for homogenization using the Omni Macro Homogenizer (Omni International, Marietta, GA, USA) equipped with a 20-mm saw tooth probe at speed 4 (~7,200 rpm) for 5 min. The homogenized samples were then transferred into a 100-mL graduated cylinder and the foam

volume measured at time 0 and after 30 min. FC and FS were determined as follows (Eq. 7 and Eq. 8):

$$FC = \frac{V_{F0}}{V_{Sample}} \times 100 \quad \text{(Eq. 7)}$$

$$FS = \frac{V_{F30}}{V_{F0}} \times 100 \quad \text{(Eq. 8)}$$

where V_{F0} is the volume of the foam at time 0 min, V_{Sample} is the initial volume of sample used (15 mL), and V_{F30} is the foam volume after 30 min. Measurements were made in duplicate, on each of the triplicate batches (n = 3) and reported as the mean ± standard deviation.

(d) Oil holding capacity (OHC) and water holding capacity (WHC). The OHC were be determined according to the method of Stone *et al.* (2015). Fermented PPEF (0.5 g) was mixed (vortexed for 10 s every 5 min for 30 min) with 5 mL of canola oil in a pre-weighed 10-mL graduated centrifuged tube. The sample was centrifuged at 11,180 × *g* for 15 min using a centrifuge (Eppendorf, Model 5810R, Mississauga, ON, Canada). The supernatant was discarded, and the tubes re-weighed. WHC were performed in a similar manner by substituting the canola oil for deionized water. OHC or WHC values were determined as the weight change in fermented protein samples after decanting (wet protein flour weight minus dry protein flour weight) relative to the dry protein flour weight (0.5 g). Measurements were made in duplicate, on each of the triplicate batches (n = 3) and reported as the mean ± standard deviation.

3.2.7 Bioactive properties

(a) Trypsin inhibitory activity. Trypsin inhibitory activity was determined according to the AOAC method 22-40.01, using UV/visible spectrophotometer. In brief, 0.25 g of fermented PPEF and 25 mL of 0.01 N NaOH was placed into a centrifuge tube and vortexed for 1 min (AOAC, 2005). The tube was then centrifuged at 14,000 × *g* for 10 min at 4ºC using a 5804R centrifuge (Eppendorf, Hamburg, Germany). The aliquots (0, 0.6, 1.0, 1.4, 1.8 mL) of the diluted supernatant was pipetted into test tubes and adjusted to 2.0 mL with deionized water. Following this, the tubes were incubated with 2 mL of trypsin solution (4 mg of trypsin in 200 mL of 0.001 M HCl) in a 37ºC water bath (VWR Scientific Products, Radnor, PA, USA) for 5 min. Five milliliters of pre-

warmed Na-benzoyl-D, L-arginine 4 nitroanilide hydrochloride (DL-BAPNA) was added to the tubes with 1 mL of dimethyl sulfoxide and diluted by Tris buffer (0.05 M, pH 8.2) to 100 mL. The samples were incubated for 10 min and then 1 mL of 30% acetic acid solution was added to the solution to terminate the reaction. The samples were filtered through Whatman No. 2 paper. One trypsin inhibitor unit (TIU) is equivalent to an increase of 0.01 absorbance unit at 410 nm per 10 mL of reaction mixture compared to the blank sample. The blank was prepared for each sample concentration with acetic acid addition before the trypsin solution. The trypsin inhibitory activity was calculated using Eq. 9, below:

$$\left(\frac{TIU}{mg\ sample}\right) = \left(\frac{TIU}{mL\ of\ extract\ taken}\right) \times \left(\frac{25\ mL\ of\ extract}{500\ mg\ of\ sample}\right) \times DF \times \left(\frac{100\%}{100\% - MC}\right) \qquad \text{(Eq. 9)}$$

where TIU is the trypsin inhibitor unit, DF is the dilution factor, MC is the moisture content of PPEF samples. Measurements were made in triplicate, on the composite batch sample (n = 3), and reported as the mean ± standard deviation.

(b) Chymotrypsin inhibitory activity. The chymotrypsin inhibitory activity of fermented PPEF was determined according to Makkar *et al.* (2007). In brief, 1 g of PPEF was placed in a 50 mL centrifuge tube with 10 mL of borate buffer (0.1 M, pH 7.6). The sample was then vortexed for 1 min and then placed on a mechanical stirrer at 500 rpm for 1 h. The sample was centrifuged at 3,000 × g for 10 min at 4ºC using a 5804R centrifuge (Eppendorf, Hamburg, Germany). In test tubes, 0, 0.25, 0.5 and 0.75 mL of sample was diluted to 1 mL with borate buffer followed by incubation at 37ºC for 10 min with 1 mL of chymotrypsin stock solution. Chymotrypsin stock solution was prepared by mixing 4 mg of chymotrypsin in 100 mL of 0.001 M HCl. A casein solution was prepared using 1 g of casein, in 100 mL of borate buffer, pH adjusted to 7.6 followed by incubation at 37ºC. Then, 2 mL of the pre-warmed casein solution was added to the solution and incubated for 10 min. The reaction was terminated by the addition of 6 mL of 18% trichloroacetic acid. Trichloroacetic acid was prepared by 18 g of anhydrous sodium acetate and 20 mL of glacial acetic acid, diluted to 1 L with deionized water. The solution was then cooled to room temperature (21°C-23ºC) for 30 min. After the 30 min, the solution was filtered through Whatman no. 2 paper. The absorbance of filtered sample was measured at 275 nm against the appropriate blank. The blank is composed of 6 mL of trichloroacetic acid and 2 mL of casein solution. The chymotrypsin unit is defined as the increase by 0.01 absorbance unit at 275 nm.

Chymotrypsin inhibitory activity is defined by the number of chymotrypsin units inhibited and can be expressed as CIU per milligram of the sample, as follows (Eq. 10):

$$\left(\frac{CIU}{mg\ sample}\right) = \left(\frac{CIU}{mL\ of\ extract\ taken}\right) \times \left(\frac{10mL\ of\ extract}{1000\ mg\ of\ sample}\right) \times DF \times \left(\frac{100\%}{100\% - MC}\right) \qquad \text{(Eq. 10)}$$

where DF is the dilution factor and MC is moisture content of PPEF samples. Measurements were made in triplicate, on the composite batch sample (n = 3), and reported as the mean ± standard deviation.

(c) Total phenolics content. The total phenolic compounds (TPC) of fermented and unfermented PPEF samples was determined according to Waterman and Mole (1994) using the Folin-Ciocalteu assay. In brief, 1 g of sample was extracted with 15 mL solvent (1% HCl in methanol) for 2 h, followed by centrifugation for 10 min at 1,510 × g and 25ºC to recover supernatant. Following this, 5 mL of solvent was added to the residue after removal of supernatant and vortexed every 5 min for 20 min. The supernatant was transferred to a new tube. This method was repeated 3 times and the supernatant pooled for analysis. From the pooled supernatants, 0.5 mL was combined with 2.5 mL of Folin-Ciocalteu reagent. The solution was at 25ºC for 5 to 8 min before the addition of sodium carbonate solution (20% w/v) and adjusting the volume to 50 mL with deionized water. After 2 h, the absorbance of samples was measured at 760 nm. A gallic acid standard curve was prepared using a 1 mg/mL gallic acid stock solution with diluted water to obtain concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL. Total phenolic content result was expressed as gram gallic acid equivalents per 100 g of sample on dry basis. The TPC was calculated using following equation (Eq. 11):

$$\left(\frac{g\ gallic\ acid\ equivalent}{100\ g\ of\ sample}\right) = \left(\frac{ABS - y}{a}\right) * C \qquad \text{(Eq. 11)}$$

where ABS is absorbance, C (mg/mL) is the concentration of the sample used, y is the y-intercept, a is the slope of the standard curve Measurements were made in triplicate, on the composite batch sample (n = 3) and reported as the mean ± standard deviation.

3.2.8 Protein quality

(a) Analysis of amino acids composition: The amino acid profile was determined for fermented PPEF samples from one fermentation batch at University of Manitoba (Winnipeg, MB) using Pico-Tag MT amino acid analysis system and high-performance liquid chromatography (HPLC). The determination of 15 amino acids was performed according to the method of Bidlingmeyer *et al.* (1987). Approximately 20 mg of each sample weighted into separate 20 × 150 mm screw cap Pyrex tubes, followed by the addition of 15 mL of 6 N HCl. The tubes were then flushed with N_2, followed by incubation at 110ºC for 20 h to complete acid hydrolysis. After acid hydrolysis, the individual amino acids were quantified by HPLC using the Pico-Tag amino acid analysis system (Waters Corporation, Milford, MA, USA). Tryptophan was determined following the AOAC method 988.15 (1995) with slight modification. Protein samples were hydrolyzed using 10 M NaOH and incubated in a 110ºC oven for 16 h followed by HPLC determination using reverse phase liquid chromatography with UV detection to determine tryptophan. The concentrations of sulfur-containing amino acids; methionine, and cysteine, was determined following AOAC method 985.28 (1995) using ion-exchange chromatography with modification. The cold performic acids was used for cysteine and methionine oxidation. They were kept overnight at 4ºC for the duration of the reaction. The sulfur containing amino acids were oxidized with performic acid and hydrolyzed with 6 M HCl at 110ºC for 18 h. The amino acid score was calculated as the ratio of individual amino acids in 1 g of PPEF to the FAO recommended reference protein standard (FAO, 1991). The amino acid composition of the reference protein (bovine casein) is as follows (amino acid, mg/g protein): histidine, 19; isoleucine, 28; leucine, 66; lysine, 58; methionine + cysteine, 25; phenylalanine + tyrosine, 63; threonine, 34; tryptophan, 11; valine, 35. The limiting amino acid was denoted by the lowest ratio.

(b) In vitro protein digestibility (IVPD): The *in vitro* protein digestibility (IVPD) of fermented PPEF was determined using the pH drop method involving a multi-enzyme solution to simulate the digestive environment. The multi-enzyme solution was prepared by mixing 31 mg of chymotrypsin (bovine pancreas ≥40 units/mg protein), 16 mg of trypsin (porcine pancreas 13,000-20,000 BAEE units/mg protein) and 13 mg of protease (*Streptomyces griseus* ≥15 units/mg solid) within 10 mL of deionized water. The pH of the solution was adjusted to 8.0 using 0.1 M NaOH and HCl and was stored at 37ºC. Approximately 62.5 ± 0.5 mg of protein was added to 10 mL of

deionized water. The solution was stirred for 1 h at 37ºC and pH adjusted to pH 8.0 again using 0.1 M NaOH and HCl before adding 1 mL of the multi-enzyme solution. The pH of the protein solution was monitored and recorded every 1 min for 10 min and the *in vitro* protein digestibility was calculated using the calculation below (Eq. 12):

$$IVPD = 65.66 + 18.10 * \Delta pH_{10min} \quad \text{(Eq.12)}$$

where ΔpH_{10min} refers to the change in pH from initial 8.0 to the end of the 10 min.

(c) In vitro protein digestibility corrected amino acid score (IV-PDCAAS). The IV-PDCAAS is calculated as the product of the amino acid score and *in vitro* protein digestibility data. Measurements were made in triplicate, on the composite batch sample (n = 3), and reported as the mean ± standard deviation. A composite sample was formed from a mixture of the three-triplicate fermented PPEF batches in even concentrations to obtain one sample, which was then triplicated in further measurements.

3.2.9 Statistics

All measurements were made in duplicate, on each of the triplicate batches or triplicate of the composite batch (n = 3), and reported as the mean ± standard deviation (except for the amino acid profile, which was only measured on one batch. SigmaPlot 12.0 (Systat Software, San Jose, CA, USA) was used to complete the statistical tests. A two-way and three-way analysis of variance (ANOVA) was used to test for differences within the main effects of fermentation time and the microorganism used in the fermentation, and well as its associated interaction for each variable measured.

4. RESULTS AND DISCUSSION

4.1 Effect of temperature and fermentation time on the physicochemical and functional properties of pea protein-enriched flour fermented by *Aspergillus oryzae* and *Aspergillus niger*

4.1.1 Determination of the effect of pH and degree of hydrolysis during fermentation

Initially, two temperatures of 30ºC and 40ºC were evaluated to determine which had the greater effect on fungal growth on PPEF substrate while measuring the change in pH. As well, fermentation time was evaluated from T=0 to 48 h to identify the desired degree of hydrolysis (DH) for subsequent analyses. As seen in Figure 4.1, the pH dropped over the duration of the fermentation at both temperatures (30ºC and 40ºC) and for both fungi (*A. oryzae* and *A. niger)*. The decrease in pH during fermentation of PPEF is presumably due to the ability of the fungal strains to ferment sugars to alcohol and acids as final metabolites. Paredes-Lopez *et al.* (1991) reported a similar gradual decline in substrate pH over time when evaluating the effect of *R. oligosporous* SSF on milled chickpea flour. This was attributed to carbohydrate conversion to simple sugars which are subsequently fermented into lactic acid, alcohol and carbon dioxide causing a pH drop in the fermented medium. These metabolic activities were also associated with enzymatic hydrolysis of proteins into amino acids and lower molecular weight peptides. It is assumed that a similar outcome occurred during the fermentation of PPEF in this thesis research.

Fermentation partially hydrolyzes the protein into peptides which potentially can lead to greater digestibility in human gut, and may also improve functionality of protein fractions for industrial utilization during food processing. During the process of fermentation, microorganisms produce enzymes which carry out the hydrolysis of protein. The effect of fermentations on the degree of hydrolysis (DH) of PPEF is shown in Figure 4.2. In both fungi, *A. oryzae* and *A. niger*, there was an increase in degree of hydrolysis with longer fermentation time. At temperature 30°C higher degree of hydrolysis was obtained over the same time period when compared to 40°C in both fungi samples. Limited studies have been focused on pea proteins and their functionality effected through partial proteolysis.

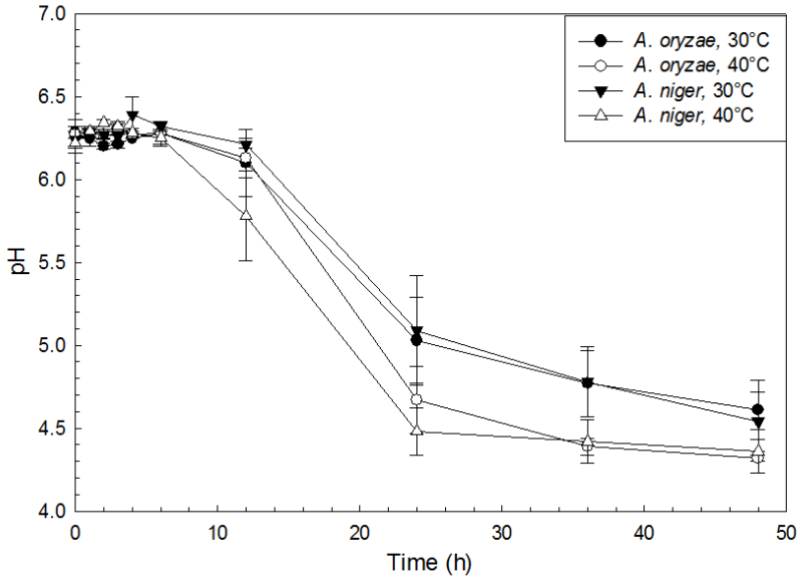

Figure 4.1 The effect of fermentation of PPEF inoculated with *A. niger* NRRL 334 and *A. oryzae* NRRL 5590 on pH over a 48-h time course.

Data represent the mean values from triplicate PPEF samples (± one standard deviation n=3). *Abbreviations:* PPEF (pea protein-enriched flour).

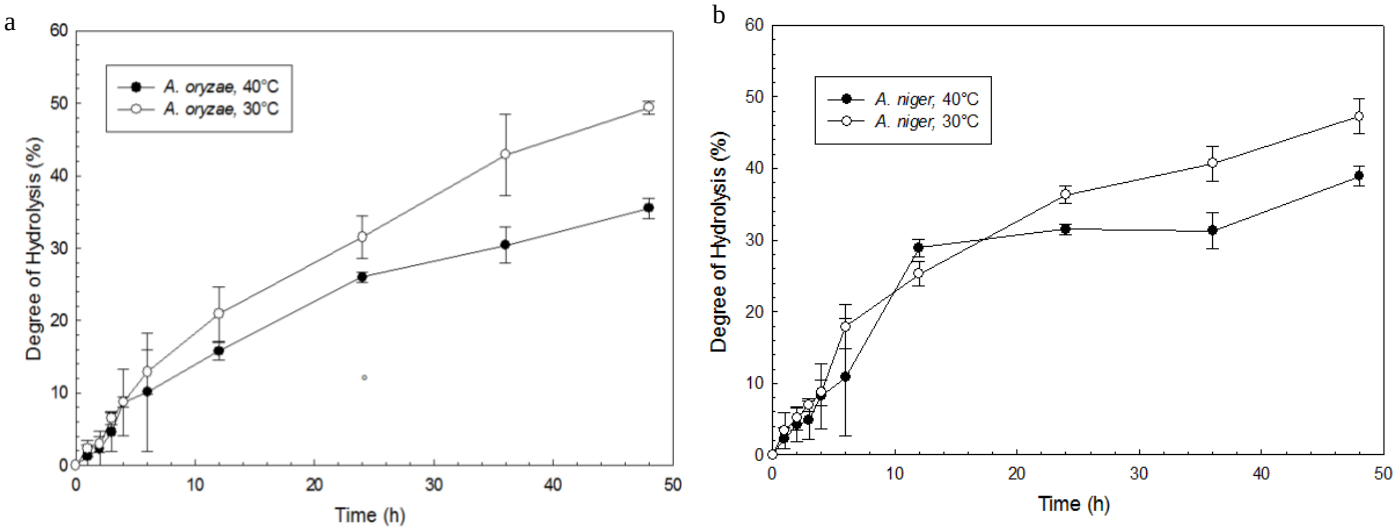

Figure 4.2 Degree of hydrolysis (%) of fermented PPEF inoculated with (a) *A. oryzae* NRRL 5590 and (b) *A. niger* NRRL 334 over a 48-h fermentation time at 30°C and 40°C.

Data represent the mean values from triplicate PPEF samples ± one standard deviation (n=3). *Abbreviations:* DH (degree of hydrolysis); *A. oryzae (Aspergillus oryzae); A.niger (Aspergillus niger);* and PPEF (pea protein-enriched flour).

Barac *et al.* (2004) stated that the ideal modification method of legume protein is through limited proteolysis. This was due to the peptides produced through partial proteolysis are of smaller molecular size and less compacted than the original proteins. Furthermore, the study indicated that controlling hydrolysis to a targeted DH could result in improved solubility, emulsification and foaming capacities. Tsumura (2005) reported that limited hydrolysis of protein (DH in the range of 2-8%) could result in enhanced functionality. Additionally, an even lower DH of between 2-4% via the endo-protease treatment of soy protein was reported to yield enhanced functional properties (Jung *et al.*, 2005; Yu *et al.*, 2007). Therefore, it is believed that maintaining a low DH is important to achieve desirable functional properties in the final product.

This attribute aided in the selection of samples for further evaluation; *Aspergillus oryzae* NRRL 5590 and *Aspergillus niger* NRRL 334 fermented at 40ºC at 0, 2, 4 and 6 h were chosen to be further evaluated. The DH were similar in trend between the two microorganisms and increased as fermentation time increased (Figure 4.2). The DH for *A. oryzae* was 10.1% after 6-h; whereas, *A. niger* was slightly higher at 10.8% after the same time course. Thus, based on the degree of hydrolysis over the 6-h period, the extent of modification of PPEF was similar. However, how specific microorganisms hydrolyze the protein (the specific modifications that occur to the peptide chain) could greatly impact the functionality and protein quality of the PPEF.

4.1.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE, in conjunction with ImageJ software, was used to analyze protein from fermented PPEF (Figure 4.3 and Table 4.1). The chromatograms shown in Figure 4.4 reveal the presence of multiple major and minor bands, reflecting the heterogenous nature of fermented PPEF. Fermented PPEF showed a high concentration of large molecular weight peptides (>60 kDa) along with a lower abundance of smaller molecular weight peptides (<30 kDa), as indicated in Table 4.1. The higher concentration of larger molecular weight peptides showed that as fermentation time increased, the structure of the protein changed. This occurred because of protein hydrolysis via microbial enzymatic activity, which cleaved PPEF proteins into smaller molecular weights and resulted in smaller-sized proteins with a decreased content of mid-to-large-sized proteins. These changes to the primary structure can be observed through quantification and observation of the changes in the chromatogram.

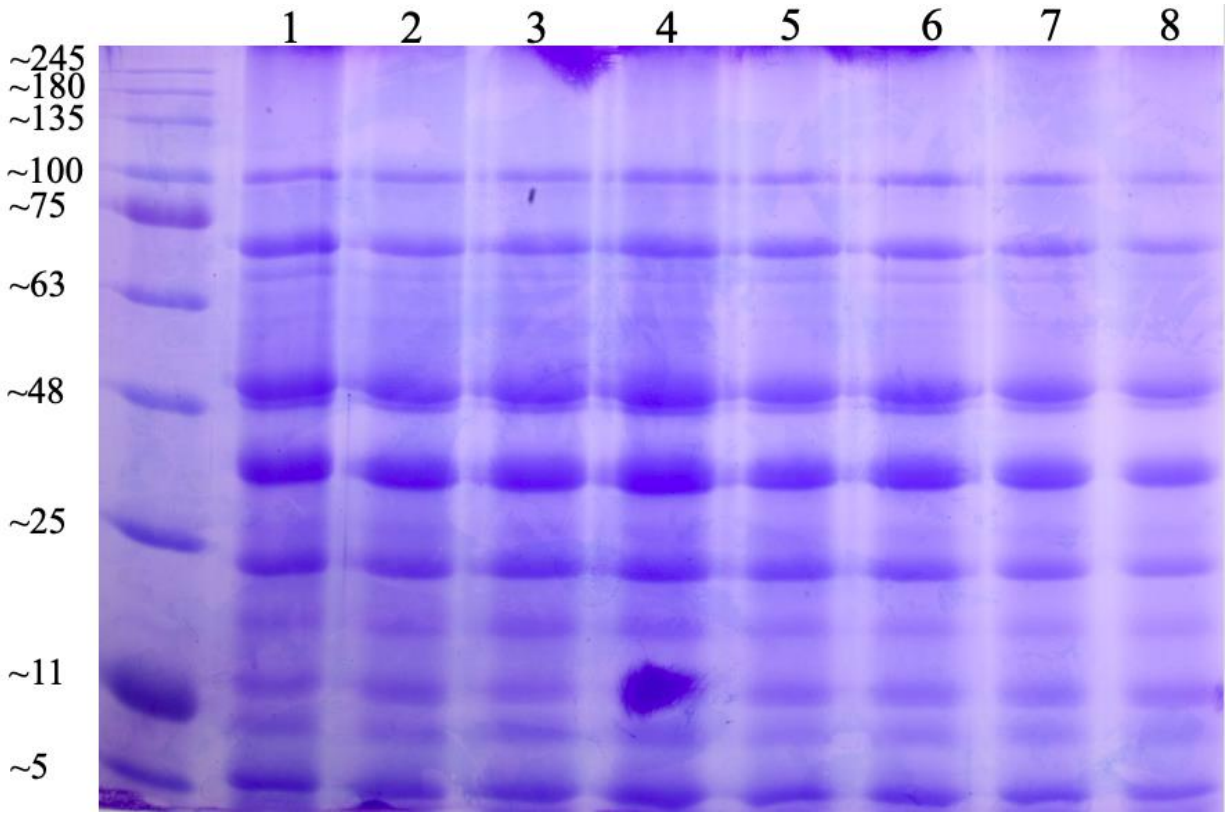

Figure 4.3 SDS-PAGE of fermented PPEF. Lanes: *A. oryzae* (1) time 0 h, (2) time 2 h, (3) time 4 h, (4) time 6 h; *A. niger* (5) time 0 h, (6) time 2 h, (7) time 4 h, (8) time 6 h.

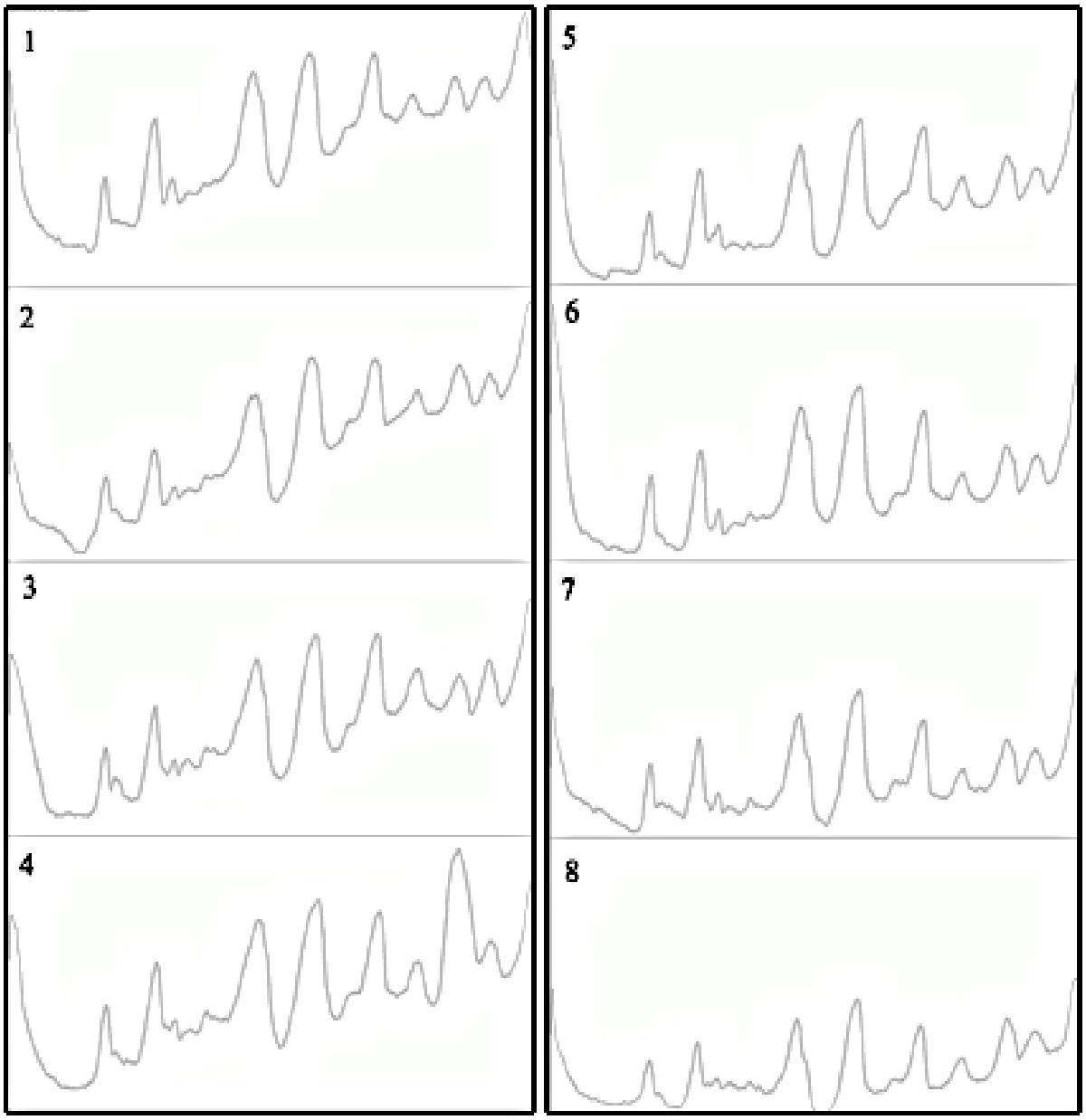

Figure 4.4 SDS-PAGE chromatogram of fermented PPEF. *A. oryzae* (1) time 0 h, (2) time 2 h, (3) time 4 h, (4) time 6 h; *A. niger* (5) time 0 h, (6) time 2 h, (7) time 4 h, (8) time 6 h.

Table 4.1 SDS-PAGE ImageJ quantification of fermented PPEF.

	Molecular weight (kDa) concentration (%)									
Sample	**~100**	**~70**	**~65**	**~48**	**~40**	**~23**	**~20**	**~17**	**~11**	**~5**
A. oryzae										
0 h	8.87	12.84	0.41	1.98	28.33	26.33	9.12	2.74	5.21	4.18
2 h	10.72	8.29	0.90	0.52	26.25	27.23	10.83	4.19	7.45	3.62
4 h	8.03	0.75	1.25	1.89	26.16	28.63	13.43	7.06	5.88	6.92
6 h	8.93	0.88	0.63	2.25	28.47	32.50	11.36	5.60	5.64	3.74
A. niger										
0 h	8.01	11.75	0.75	0.33	23.87	27.69	10.26	4.86	8.14	4.35
2 h	8.92	10.15	1.29	0.60	24.63	28.73	10.48	3.20	7.58	4.41
4 h	10.30	9.64	1.19	0.59	22.98	28.68	9.48	3.68	8.46	5.01
6 h	7.20	6.93	0.73	1.14	21.95	26.83	14.14	5.57	10.47	5.05

All data is reported as the mean ± one standard deviation.
Abbreviations: *A. oryzae (Aspergillus oryzae); and A. niger (Aspergillus niger).*

The SDS-PAGE profiles albumin protein and the three major globulin storage proteins found in pea protein, including legumin (11*S*), vicilin (7*S*) and minor amounts of convicilin (7*S*). Legumin protein has a molecular mass between 300 and 400 kDa, its acidic monomers ~40 kDa and basic monomers ~20 kDa. Vicilin proteins have a molecular mass of 150-170 kDa with each monomer ~47-50 kDa. It has known proteolytic cleavage resulting in fractions of 12-36 kDa. Convicilin has a molecular mass of ~70 kDa (Lam *et al.*, 2018). Convicilin is similar to vicilin; however, it contains sulfur amino acid resides and has no covalent bonds to saccharides (Sikorski, 2000). In soybean protein, 7*S* and 11*S* subunits had different susceptibilities to enzyme-induced hydrolysis, a feature that was attributed to protease activity. Endo-proteases are reported to have preference over hydrolyzation of vicilin over legumin due to legumin having a compact structure that is difficult for proteases to act upon (Gabriel *et al.*, 2008; Barac *et al.*, 2015). Another storage protein found in pea in small amounts is albumin. Albumin has a high molecular weight of ~50-110 kDa, it has a large fraction comprised of two polypeptides with molecular weight of ~25 kDa and a minor fraction with a low molecular weight of ~6 kDa (Dziuba *et al.*, 2014; Lam *et al.*, 2018). This protein is minor compared to globulin storages proteins.

Fermented PPEF (4 and 6 h; Lanes 3, 4, 7 and 8 in Figure 4.3) showed the greatest decrease of larger molecular weight peptides with a concurrent increase in concentration of lower molecular weight peptides when compared to content at the initial time (0 h). Specifically, 4 and 6 h SSF showed apparent reductions in the densities of the ~70 kDa bands when compared with earlier fermentation times (Table 4.1). It is also indicated that the 6-h SSF treatments showed an increase in band size/density at ~48 kDa band after fermentation and continued to increase at bands ~40 to ~5 kDa. From the SDS-PAGE gel, it can be observed that there was change in protein distribution of PPEF sample, from bands of large-sized proteins (>60 kDa) to small sized or peptides (<30 kDa), presumably due to the proteolytic activity. Peptidase systems are well-developed in microorganisms, and these enzymes have the ability to hydrolyze larger proteins into smaller peptides (Xiao *et al.*, 2018). The peptidase system used by the microorganism will define the degree and specificity of hydrolysis of proteins into smaller peptides. The modification of soybean, red bean and chickpea proteins during fermentation into smaller peptides, demonstrated using SDS-PAGE, has been reported in previous studies (Lim *et al.*, 2010; Xiao *et al.*, 2015, 2018). Specifically, Xiao *et al.* (2015) found a similar trend of large to medium sized proteins becoming

reduced to smaller molecular weight through the proteolytic activity produced by *C. militaris* SN-18 during SSF of chickpea.

4.1.3 Physicochemical properties

(a) Proximate composition

The proximate composition of the fermented pea protein-enriched flour (PPEF) composite samples are summarized in Tables 4.1.3 as a function of the microorganism strain used to inoculate the sample and fermentation time. The samples were made from fermenting in triplicate batches at various hours (0, 2, 4 and 6), freeze-dried, ground into powder and combined into a composite sample for proximate analysis. Overall, protein levels were found to increase 5% and 15%, lipid content decreased 49% and 94%, and ash content remained at the same concentration (~4.7%) over the course of fermentation in *A. oryzae* and *A. niger* samples, respectively. PPEF at the initial time of fermentation was composed of 47% of protein, 4.7% of ash and 1.5-1.7% of lipid content. The higher amounts of protein content can be attributed to the production of fungal proteins as well as, the utilization of nutrients in PPEF. It is presumed that the increase in protein content is attributed to the fungi utilizing lipids and starch, due to the utilization the fungi is able to produce proteins and grow. During fermentation, it is hypothesized that fungi secreted extracellular enzymes that were able to dissolve nutrients such as simple sugars and fatty acids, therefore, decreasing the lipid and carbohydrate content in PPEF.

A two-way analysis of variance (ANOVA) found the interaction between microorganism and fermentation time to be significant for protein and lipid ($p<0.05$), but not for ash ($p>0.05$) (Table 4.2). Due to this, both fungal inoculant and fermentation time will be discussed separately for ash. Protein content increased with duration of fermentation for both fungal samples with the maximum 6-h values resulting in 49.2% for *A. oryzae* and 53.3% for *A. niger* PPEF. As the fermentation time increased from 0 to 6-h the lipid levels decreased for both *A. oryzae*- and *A. niger*-fermented samples, reducing by 49% and 94%, respectively (Table 4.3). The higher amounts of protein content can be attributed to the enzyme secretion from the fungi used in the fermentation. Since the fermentation was a short-term with a maximum of 6 h, the fungi is utilizing nutrients from the substrate in order to grow and secrete enzymes. It is presumed that the increase in protein content is attributed to the fungi utilizing lipids and carbohydrates, due to the utilization the fungi is able to produce proteins.

Table 4.2 Two-way and three-way analysis of variance of the composition, surface and functional properties of fermented PPEF.

	Composition[1]			Surface properties[1]		Functional properties[2]						
	Protein	**Ash**	**Lipid**	**Charge**	**Hydro-phobicity**	**Sol**	**WHC**	**OHC**	**EA**	**ES**	**FC**	**FS**
Main effects												
Fungi	p<0.05	NS	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001	NS	p<0.01	p<0.001	p<0.001
Time	p<0.001	NS	p<0.05	p<0.05	p<0.001	p<0.001	p<0.001	p<0.001	NS	p<0.001	p<0.001	p<0.001
pH	-	-	-	-	-	p<0.001	-	-	p<0.001	p<0.001	p<0.001	p<0.001
Interactions												
Fungi × time	p<0.001	NS	NS	p<0.05	p<0.001	p<0.001	p<0.001	p<0.001	p<0.05	p<0.001	p<0.001	p<0.001
Time × pH	-	-	-	-	-	p<0.001	-	-	p<0.05	p<0.001	p<0.001	p<0.001
Fungi × pH	-	-	-	-	-	p<0.001	-	-	p<0.05	p<0.01	p<0.001	p<0.001
Fungi × time × pH	-	-	-	-	-	p<0.001	-	-	p<0.001	p<0.01	p<0.001	p<0.001

[1]Measurements were performed in triplicate on the composite sample blend from three batches of fermentation (n=3).
[2]Data represent the mean of triplicate measurements on flour ± one standard deviation (n = 3).
Conditions:
Fungi (*Aspergillus oryzae* and *Aspergillus niger);* Time (0, 2, 4, and 6 h)*;* pH (3.0, 5.0 and 7.0)
Abbreviations: Sol (solubility); WHC (water hydration capacity); OHC (oil holding capacity); EA (emulsion activity); ES (Emulsion stability); FC (foaming capacity); FS (foaming capacity); PPEF (pea protein-enriched flour); (-) (Not applicable); and NS (Not significant).

Table 4.3 Proximate composition of fermented PPEF.

Sample	Protein (%, d.b.)	Ash (%, d.b.)	Lipid (%, d.b.)
A. oryzae			
0 h	46.80±0.94	4.70±0.18	1.74±0.13
2 h	48.33±0.88	4.79±0.14	1.73±0.17
4 h	48.89±0.90	5.11±0.09	1.31±0.33
6 h	49.18±0.81	4.66±0.23	0.88±0.32
A. niger			
0 h	46.51±0.60	4.68±0.04	1.48±0.14
2 h	46.30±0.80	4.69±0.57	1.78±0.68
4 h	51.08±0.18	4.77±0.05	0.50±0.27
6 h	53.34±0.69	4.71±0.07	0.09±0.02

Data represents the mean values from triplicate batches of composite blend PPEF samples ± one standard (n=3).
Abbreviations: PPEF (pea protein-enriched flour); *A. oryzae (Aspergillus oryzae); A. niger (Aspergillus niger);* and d.b. (dry weight basis).

In literature, an increase in protein content through SSF of flours were reported (Akubor & Chukwu, 1999; Adebowale & Maliki, 2011; Difo *et al.*, 2015; Xiao *et al.*, 2015; Xiao *et al.*, 2018). However, the increase in protein content was attributed to the increase in fungal biomass that was produced from the fermenting microorganism used in SSF.This could be occurring in these fermentation systems due to longer fermenting time in comparison to the PPEF. Whereas, the PPEF was fermenting for 6 h with the main focus on utilizing nutrients and producing enzymes.

The ash content was maintained at 4.7% in both inoculated PPEF samples. Over the 6-h SSF, there was no significant interaction between the fermentation time and microorganism (Table 4.2). The decrease in lipids instead of minerals and protein potentially occurred because of the readily available lipids for utilization by fungi. Filamentous fungi secrete enzymes that work to hydrolyze the protein-lipid complexes, freeing the lipids from the complex. The free lipids provide a source of nutrients for the fungi to utilize and grow, ultimately leading to a reduction in lipid content (Adebowale & Maliki, 2011). Therefore, as protein levels increased, the ash levels remained the same and lipids decreased in the fermented PPEF.

Reductions in parameters such as, lipids, ash, carbohydrates have been reported in literature and attributed to the metabolism by the microorganisms in the fermentation medium to increase growth and from biomass production (de Reu *et al.*, 1995; Akubor & Chukwu, 1999; Adebowale & Maliki, 2011; Difo *et al.*, 2015; Xiao *et al.*, 2015, 2018). Furthermore, the microbes used in SSF are able to produce many enzymes, including: proteases, amylases, lipases, esterases and celluases, that hydrolyze proteins, polysaccharides and lipids. The hydrolysis of these components could contribute to the development of food products with enhanced nutrition, flavour and aroma (Xiao *et al.*, 2017). Specifically, *A. oryzae* has been reported to secrete large amounts of hydrolytic enzymes including protease, amylase, cellulase and phytase (Chawla *et al.*, 2017).

(b) Surface charge (zeta potential)

The surface charge, or zeta potential (ZP) values for fermented PPEF inoculated with *A. oryzae* and *A. niger* are shown in Table 4.4. The surface charge is important in indicating the repulsion or attraction of particles, and thus reflects the stability of the fermented PPEF in solution. A high ZP (less than -30 mV or more than +30 mV) indicates greater stability, in contrast to a low ZP (in-between -30 mV and +30 mV) (Wu *et al.*, 2015). At pH values away from the pI, proteins carry a high charge leading to electrostatic repulsion, preventing the aggregation of proteins and improving solubility of proteins in the solution due to protein-favoring water interactions (Can Karaca *et al.*, 2011). Moreover, a high negative ZP has been reported to be due to a low ratio of basic to acidic amino acid clusters (Tirgar *et al.*, 2017). The surface charge was evaluated at pH 7.0 for all PPEF samples; thus, it would be expected to cause repulsion of particles. The interaction between fermentation time and inoculant (either *A. oryzae* and *A. niger*) in the sample were found to be significant ($p>0.05$) (Table 4.2). The ZP ranged from -16.20 mV (0 h) to -18.43 mV (6 h) in *A. oryzae*-inoculated PPEF sample; whereas, *A. niger*-inoculated PPEF samples ranged from -13.53 mV (0 h) to -18.57 mV (6 h) over the same fermentation period (Table 4.4). Due to the solvent (pH 7.0) having a charge greater than the pea globulin pI of ~4.6, the resultant material would carry a net-negative charge for all PPEF samples. The surface charge (mV) for samples increased with fermentation time, an observation that was attributed to the fungi producing protease enzymes that hydrolyzed the protein, exposing hydrophilic moieties and revealing a higher proportion of charged amino acids at the PPEF surface.

Table 4.4 Physicochemical properties of fermented PPEF inoculated with *A. oryzae* and *A. niger* measured at pH 7.0.

Sample	Zeta potential (mV)	Surface hydrophobicity (a.u.)
A. oryzae		
0 h	-16.20±0.80	14.14±0.48
2 h	-14.30±0.26	13.01±0.86
4 h	-15.87±0.96	12.31±0.98
6 h	-18.43±0.31	8.37±0.72
A. niger		
0 h	-13.53±0.46	21.63±1.59
2 h	-15.00±0.72	19.81±0.49
4 h	-14.83±0.51	13.26±0.68
6 h	-18.57±0.95	13.86±0.83

Data represents the mean values from triplicate batches of composite blend PPEF samples ± one standard (n=3).
Abbreviations: PPEF (pea protein-enriched flour); *A. oryzae (Aspergillus oryzae); A. niger (Aspergillus niger);* mV (millivolts) and a.u. (arbitrary units).

Additionally, the lower ZP charge may be due to the pH of the solution (pH 7.0); if it was further away from the pI the stability would be greater. Thus, weak repulsive forces between the proteins enabled protein-protein aggregation to preferentially-occur compared with protein-water interactions in the fermented PPEF samples. The PPEF solution was shown to be less stable at T=0 (before fermentation) and increasing in stability over the fermentation time course for both fungal strains. Exploring the surface charge of fermented flours is novel and thus no true comparisons exist for comparison.

However, the results of this study were found to be similar values reported in literature for protein isolates. Tirgar *et al.* (2017) found pea protein concentrate to have a ZP of -15.96 which was within range determined for the fermented PPEF samples. Additionally, Can Karaca *et al.* (2011) reported pea protein isolates prepared by isoelectric precipitation at pH 7.0 had a ZP of -21 mV, a value that was slightly-lower than when the isolates were prepared via salt extraction (-20.9 mV).

However, these previous results were slightly higher than the PPEF samples, an observation that could be attributed to the pea protein being a protein-enriched flour.

(c) Surface hydrophobicity

Hydrophobicity is the affinity of non-polar solutes to adhere to one another in aqueous environments, thus hydrophobic sites on the protein can determine the conformation and amount of protein-protein interactions that occur (Cardamone & Puri, 1992). Surface hydrophobicity measurements specify the degree of protein unfolding in the solution, as well as the conformation of surface hydrophobic "patches" of the protein (Wang *et al.*, 2014). Exposed hydrophobic sites contribute to the functionality properties of the protein (i.e., solubility, oil holding, emulsification and foaming), and are essential to evaluate when making a food ingredient. Aromatic amino acids such as tyrosine, histidine and phenylalanine give rise to greater surface hydrophobicity due to their non-polar side-chains. Surface hydrophobicity of all fermented PPEF at pH 7.0 are shown in Table 4.4. The interaction between inoculant and fermentation time were found to be statistically significant ($p<0.001$) (Table 4.2). The surface hydrophobicity decreased in PPEF from 14.14 to 8.37 a.u. (arbitrary unit) and from 21.63 to 13.86 a.u., when inoculated with *A. oryzae* and *A. niger,* respectively (Table 4.4). It is hypothesized that during SSF, hyphae was formed and was hydrophilic in nature. In literature, the hyphae surface hydrophobicity was shown to be variable. During fungal adaptation and attachment, the hyphae surface hydrophobicity was interchangeable from hydrophilic to hydrophobic (Vujanovic & Kim, 2017). Thus, hyphae could be hydrophilic in nature during the short duration of PPEF fermentation during which the fungus could be adapting to the environment. Additionally, fungal surface hydrophobicity is known to be variable among species, even within species, and in this case the age of fungus and composition of the growth medium are factors that could affect the surface hydrophobicity (Bigelis *et al.*, 2006). Thus, the differences between the *Aspergillus* strains studied could be attributed to these types of effects. Overall, the fermented PPEF is hydrophilic because of the fungal hyphae, but at the protein level, the surface is hydrophobic due to the proteolytic activity of the fungi. Specifically, fungal enzymes produced during SSF hydrolyzed the PPEF proteins, exposing their hydrophobic core. In turn, and like surface charge, the surface hydrophobicity was affected and decreased since the hydrophobic proteins favored hydrophobic interactions, thus forming an aggregate.

There is limited available literature on the surface hydrophobicity of fermented enriched

flours. However, there are a number of studies on pea protein isolates and concentrates (Can Karaca *et al.*, 2011; Tirgar *et al.*, 2017). Can Karaca *et al.* (2011) reported pea protein isolate surface hydrophobicities of 84.76 a.u. and 77.83 a.u. prepared by isoelectric precipitation and salt extraction, respectively. Tirgar *et al.* (2017) found the surface hydrophobicity of pea protein concentrates to be 68.47 a.u., which was higher than determined for fermented PPEF samples at all fermentation times for either fungal strain. The surface hydrophobicity values of both isolate and concentrate pea protein from the Tirgar *et al.* study were considerably higher than fermented PPEF samples, which could be attributed to the protein being extracted and processed differently. Surface hydrophobicity is highly important for emulsifying properties due to the absorption at the surface of oil droplets through electrostatic forces and steric interactions (Tirgar *et al.*, 2017). Due to the low surface hydrophobicity of fermented PPEF, the emulsification potential could affect the functionality of this material as a potential food ingredient.

Another consideration when evaluating surface hydrophobicity is that the ANS-binding method may not accurately reflect surface hydrophobicity due to the strong hydrophobic interactions that occur between the protein monomers, which shield the hydrophobic amino acid residues, yielding lower results (Hiller and Lorenzen, 2008). This could occur due to the partial unraveling of peptides as a consequence of fermentation which exposes the hydrophobic moieties. With the increased exposure of hydrophobic groups, aggregation of peptides through hydrophobic interactions would occur and the large, resultant aggregate structure would re-bury the hydrophobic groups within the interior (Jung *et al.*, 2005). Peptides being cleaved into individual amino acids during fermentation via proteolytic activity could also influence hydrophobicity results. Amino acid-mediated steric hindrance could prevent its ability to bind with the ANS probe, resulting in a lower measured hydrophobicity value.

4.1.4 Functionality

(a) Nitrogen solubility

The nitrogen solubility of fermented PPEF was measured at three different pH values (3.0, 5.0, 7.0) are summarized in Table 4.5. Protein solubility is the amount of nitrogen contained in a protein that is able to be dissolved in an aqueous solution (Adler-Nissen, 1976). Protein solubility is an important parameter since it influences other functional properties, specifically foaming and emulsification (Kinsella, 1976). If the protein is highly-soluble, the potential range of food

applications increases. Hydrophilic interactions determine whether the protein would stay in solution because protein-solvent interactions would be favored over protein-protein interactions. Additionally, pH has a major influence on solubility; pH values above and below the pI of the protein would lead to increasing electrostatic repulsive forces, thereby favoring protein-solvent interactions. At pH values closer to the protein's pI, protein-protein interactions would be favored, leading to aggregation of proteins, and thus, greater insolubility (Wu *et al.*, 1998). A three-way ANOVA determined that fermentation time, the fungal inoculant and pH had a significant effect on protein solubility ($p<0.001$) (Table 4.2). All fermented samples (*A. oryzae* and *A. niger)* were shown to decrease solubility over fermentation time (0-6 h) at all pH values (3.0, 5.0 and 7.0), as shown in Table 4.5. The statistical analysis revealed that interactions between the fungal strains and fermentation time, fungal strains and pH, fermentation time and pH were all significant ($p<0.001$) (Table 4.2). The reduction in solubility by fermentation was attributed to the fungi producing proteolytic enzymes which hydrolyzed protein into smaller peptides, exposing hydrophobic and hydrophilic moieties on the protein. Protein solubility is highly-dependent on the hydrophilic or hydrophobic properties that become exposed on the protein.

The hydrophilic peptides on proteins potentially had a greater susceptibility to proteolytic activity and due to the proteolytic activity unraveling of the protein occurred exposing hydrophobic moieties. The hydrophobic moieties allowed for protein-protein association over protein-solvent interactions, and thus aggregation occurred. The surface charge of the fermented PPEF remained low, ranging from -13 to -18 mV at pH 7.0; whereas, surface hydrophobicity decreased with fermentation time hypothetically due to large aggregate formation. The production of fungal enzymes exposed hydrophobic entities that favored protein-protein interactions. Additionally, the solutions were left to hydrate for 16 h, allowing time for the hydrophobic regions on the protein surface to aggregate together and fall out of solution. Overall, the PPEF nitrogen solubility was found to be greatest at pH 7.0 (~58 % to ~49%), followed by pH 3.0 (~43 % to ~31%); whereas, the protein was least soluble at pH 5.0 (~22 % to ~19%). The lower protein solubility of PPEF at pH 5.0 occurred due to its closeness to the pI of pea protein (pI= 4.6). Most pulse proteins have a pI between pH 4.0 and 6.0 (Boye *et al.*, 2010b). The high net charge favors protein-solvent interactions over protein-protein interactions, reducing the aggregation and precipitation of protein. A similar effect of pH dependency was found in fermented African oil bean seed flour (Akubor & Chukwu, 1999). In fermented African oil bean seed flour, a minimum and maximum nitrogen

solubility was observed at pH 4.0 and pH 8.0, respectively. Thus, the solubility was greater at alkaline than acidic pH values. The fermented flour was found to have a lower nitrogen solubility as fermentation time continued. The increase in nitrogen solubility was attributed to proteolytic activity in fermented seeds yielding peptides and free amino acids in water (Akubor & Chukwu, 1999). A similar phenomenon is presumed to have occurred in PPEF fermented medium, where proteolytic activity exposed more hydrophobic moieties, increasing the surface hydrophobicity and increase protein-protein interactions.

Table 4.5 Nitrogen solubility of fermented PPEF at pH values of 3.0, 5.0 and 7.0.

	Nitrogen solubility (%)		
Sample	**pH 3.0**	**pH 5.0**	**pH 7.0**
A. oryzae			
0 h	39.31±0.86	20.63±0.88	55.38±0.39
2 h	32.99±0.62	26.91±1.00	54.97±0.48
4 h	32.83±0.81	25.22±0.92	54.36±0.99
6 h	31.63±0.79	19.54±0.87	51.97±0.64
A. niger			
0 h	43.07±0.88	22.27±0.64	58.31±0.54
2 h	42.86±0.61	22.74±0.96	56.78±0.37
4 h	36.35±0.63	20.01±0.45	51.26±0.95
6 h	31.28±0.60	19.25±0.65	49.92±0.77

Data represent the mean of triplicate measurements on flour ± one standard deviation (n = 3).
Abbreviations: PPEF (pea protein-enriched flour); *A. oryzae (Aspergillus oryzae); A. niger (Aspergillus niger).*

(b) Emulsifying properties

Emulsification activity and stability are important functional properties when considering potential ingredients for incorporation in various food systems since proteins are able to be used as emulsifiers to help stabilize oil-water interface and reduce interfacial tension. The emulsifying activity (EA) and emulsion stability (ES) were determined for fermented PPEF at pH values of 3.0,

5.0 and 7.0, as shown in Table 4.6. The EA represents the height of the emulsion along with the ability to prevent flocculation and coalescence in the food system; whereas, the ES is the ability of the emulsion to resist changes to its structure over time. Emulsifying properties are strongly influenced by the flexibility of solutes and exposure of hydrophobic moieties of the protein (Ahmed *et al.*, 2011). A three-way ANOVA analysis of the fermented PPEF found that fermentation time, fungal strain and pH had a statistically-significant effect on EA ($p<0.001$) (Table 4.2). The effect of fermentation time and fungal strain interaction was dependent upon the pH used ($p<0.05$). It was also found that there was a significant interaction between the fermentation time and fungal strain at pH 3.0 ($p<0.001$) and pH 7.0 ($p<0.001$) (Table 4.2). Thus, pH had an effect on the EA; the farther away from the pI of the protein, the greater the resultant EA. Specifically, the EA at pH 5.0 had low values, this could be attributed to nearness to the pI of protein in fermented PPEF. EA values of fermented PPEF inoculated with *A. oryzae* were 35.2%, n.d. (none detected) and 40.5% after 6 h of fermentation at pH values of 3.0, 5.0 and 7.0, respectively. Whereas, EA values of fermented PPEF inoculated with *A. niger* resulted in similar values of 35.5%, n.d. and 37.9% after 6 h of fermentation at pH values of 3.0, 5.0 and 7.0, respectively (Table 4.6). The ES of fermented PPEF was shown to maintain stability over 6 h of fermentation. There was a significant interaction between fermentation time, fungi genus and pH ($p<0.01$) (Table 4.2). The ES at pH 3.0 increased slightly from 98.5% to 99.0% and 97.3% to 99.4%, n.d. at pH 5.0 and at pH 7.0 ranged from 97.6% to 94.9% and 94.4% to 94.8% for *A. oryzae* and *A. niger*, respectively (Table 4.6).

It was shown that shifting the pH away from the pI of the protein (pH of 4.6) enhanced the emulsifying properties of fermented PPEF. In comparison, fermented chickpea flour showed acidic pH had the lowest EA and ES; whereas, the highest EA and ES was at alkaline pH (Xiao *et al.*, 2015). Xiao *et al.* (2015) attributed this to the pH being farther away from the pI, which lead to the enhancement of emulsification properties. Fungal fermentation enables modification of protein, exposing hydrophobic groups and allowing the protein to interact with the oil-water interface. The emulsification activity and stability could be attributed to the fungal fermentation producing hyphae under wet conditions, thus making the substrate hydrophilic in nature. At the protein level, it could be hypothesized that there are hydrophobic properties due to the exposure of moieties through protease activity of the fungi.

Table 4.6 EA and ES of fermented PPEF at pH 3.0, 5.0 and 7.0.

	EA (%)			ES (%)		
Sample	**pH 3.0**	**pH 5.0**	**pH 7.0**	**pH 3.0**	**pH 5.0**	**pH 7.0**
A. oryzae						
0 h	35.88±0.39	n.d.	38.57±0.68	98.48±0.10	n.d.	97.62±0.76
2 h	34.50±0.25	n.d.	38.44±0.68	96.06±0.23	n.d.	94.83±0.96
4 h	36.31±0.59	n.d.	38.74±0.87	98.62±0.85	n.d.	95.53±0.60
6 h	35.15±0.34	n.d.	40.52±0.87	98.98±0.52	n.d.	94.89±0.66
A. niger						
0 h	36.03±0.70	n.d.	38.14±0.74	97.34±0.33	n.d.	94.38±0.67
2 h	35.83±0.25	n.d.	38.92±0.65	97.06±0.89	n.d.	94.88±0.46
4 h	35.15±0.61	n.d.	38.90±0.91	98.06±0.86	n.d.	94.63±0.18
6 h	35.46±0.10	n.d.	37.89±0.12	99.42±0.78	n.d.	94.80±0.27

Data represent the mean of triplicate measurements on flour ± one standard deviation (n = 3). *Abbreviations:* EA (emulsifying activity); ES (emulsifying stability); PPEF (pea protein-enriched flour); *A. oryzae (Aspergillus oryzae); A. niger (Aspergillus niger);* and n.d. (none detected).

The hydrophobic moieties that are exposed aggregate together since the protein solution is stirred for 16 h to increase homogeneity of solution. Over this amount of time, the proteins are able to re-orient, forming large aggregates that then fall out of solution. In contrast, Chawla *et al.* (2017) found SSF with *A. oryzae* improved the emulsifying properties of black-eyed pea flour samples. The authors attributed this to fungal proteolytic activity exposing hydrophobic groups altering the hydrophilic-lipophilic balance, favoring emulsification. Stirring for 16 h to increase the solubility of protein but may have had a negative effect on emulsifying properties of PPEF. As discussed in section 4.4.1, solubility of PPEF decreased with increase fermentation time. Proteolytic activity produced from the fungus exposed the hydrophobic moieties, the solution was then stirred before measuring activity. Therefore, it's hypothesized an aggregate formed which precipitated out before measuring emulsification properties. Solubility is an important factor that can affect emulsification properties, as proteins solubility is known to be pH-dependent. Soluble proteins are intrinsically surface active due to their amphiphilic nature which allows their absorption at oil-water interfaces (Prinyawiwatkul *et al.*, 1997). If there is less-soluble proteins, absorption could be inhibited leading to less solubility and consequently low emulsion activity and stability values.

Physicochemical properties such as surface hydrophobicity and surface charge of proteins has been reported as important factors that can influence the EA and ES properties due to their adsorption and flexibility of proteins at the interface (Shevkani *et al.*, 2015; Ghumman *et al.*, 2016; Xiao *et al.*, 2018). Xiao *et al.* (2018) hypothesized that fungal SSF of red bean protein increased EA due to increased hydrophobicity of proteins. In the case of fermented PPEF, the surface hydrophobicity was shown to decrease over fermentation time, thus the EA did not increase possibly due to the protein's inflexibility and low adsorption at the oil-water interface. The surface charge of fermented PPEF increased over fermentation time; however, the results were in the low range (between -30 mV and +30 mV) and consequently samples would be unstable due to the lack of repulsion and have an inclination to flocculate (Ghumman *et al.*, 2016).

Fermented proteins contain a lower molecular mass due to the hydrolysis into peptides which allows for the ease of migration at the oil-water interface (Lim *et al.*, 2010; Xiao *et al.*, 2018). The ease of migrating at the oil-water interface would in turn increase the EA. Xiao *et al.* (2015) evaluated the extent of hydrolysis by SSF using SDS-PAGE, finding protein composition of fermented red bean flour to be hydrolyzed to a greater extent resulting in greater EA because of smaller peptides ease of migration at the oil-water interface. When evaluating the SDS-PAGE of fermented PPEF (Figure 4.3), there were slight protein modifications that occurred over the fermentation period; however, when compared to the extent of protein modification by Xiao *et al.* (2015), their study found the peptides were more extensively hydrolyzed. Hydrolysis was shown to occur mainly at 70 kDa in fermented PPEF; whereas, Xiao *et al.* (2015) showed hydrolysis at multiple molecular weights. It is hypothesized that the fermented PPEF was not extensively hydrolyzed at all molecular weights, making migration at the interface difficult due to the higher molecular weight causing the lower rate of EA.

(c) Foaming properties

Foaming capacity (FC) and stability (FS) of fermented PPEF as a function of pH is given in Table 4.7. Foams are used in food to improve the texture, consistency and overall appearance of foods. FC is the maximum volume increase due to dispersed proteins, i.e., how much air becomes entrapped within the protein matrix (Sathe *et al.*, 1982). FS is the ability of the foam to remain the same over a period of time (Wouters *et al.*, 2016). A three-way analysis of variance found FC had a statistically-significant interaction between fermentation time, fungi and pH

($p<0.001$) (Table 4.2). Therefore, the main effects cannot be discussed separately. FC had a trend of decreasing over the fermentation time, resulting in the lowest capacity at time 6-h, FC of PPEF samples fermented by *A. oryzae* and *A. niger* became reduced from 170% to 130% and 166% to 132% at pH 3.0, from 138% to 108% and 144% to 111% at pH 5.0, and from 174% to 153% and 184% to 164% at pH 7.0, respectively (Table 4.7). The reduced FC may be attributed to the lower solubility of the samples, which follow the same trend of decreasing over fermentation time. At pH 7.0, the greatest FC and solubility was observed; whereas, PPEF at pH 5.0 had the lowest FC and solubility. These parameters affect the ability of the emulsion protein to reach the air-water interface, thereby reducing the FC%.

Table 4.7 Foaming capacity (FC%) and stability (FS%) of fermented PPEF at 3.0, 5.0 and 7.0.

	Foaming capacity (%)			**Foaming stability (%)**		
Sample	**pH 3.0**	**pH 5.0**	**pH 7.0**	**pH 3.0**	**pH 5.0**	**pH 7.0**
A. oryzae						
0 h	170±9	138±7	174±4	15±1	n.d.	75±3
2 h	154±10	132±13	173±12	11±1	n.d.	52±3
4 h	151±12	124±8	157±7	9±1	n.d.	44±3
6 h	130±9	108±2	153±13	4±1	n.d.	23±3
A. niger						
0 h	166±5	144±4	184±16	14±3	n.d.	86±7
2 h	151±7	133±9	171±16	7±1	n.d.	61±4
4 h	142±13	114±4	167±5	4±1	n.d.	50±5
6 h	132±8	111±10	164±4	4±0	n.d.	21±1

Data represent the mean of triplicate measurements on flour ± one standard deviation (n = 3).
Abbreviations: PPEF (pea protein-enriched flour); *A. oryzae (Aspergillus oryzae); A. niger (Aspergillus niger);* and n.d. (none detected).

A three-way ANOVA found FS also had a statistically-significant interaction between fermentation time, fungi and pH ($p<0.001$) (Table 4.2). Because of this, the main effects will not be discussed separately. All fermented PPEF samples followed a similar trend to that of FC in terms of reducing stability with increased time of fermentation. The FS had a trend of decreasing

over the fermentation time, resulting in the lowest stability at time 6-h; FS of PPEF samples fermented by *A. oryzae* and *A. niger* became reduced from 15% to 4% and 14% to 4% at pH 3.0, none was detected at pH 5.0, and reduced from 75% to 23% and 86% to 21% at pH 7.0, respectively (Table 4.7). The limited foaming properties could be attributed to the compact structure and low solubility of the fermented PPEF. Compact structure is not able to re-orientate and form film around the bubble.

Previous studies have evaluated the effect of SSF on FC and FS of legume and cereal flours (Elkhalifa, 2005; Adebowale & Maliki, 2011; Chandra-Hioe *et al.*, 2016; Chawla *et al.*, 2017; Xiao *et al.*, 2018). Xiao *et al.* (2018) reported no significant difference in FC of fermented red bean flour (RBF) compared to unfermented red bean flour; whereas, FS of fermented RBF was found to be higher in than the unfermented sample. This was attributed to structural changes of proteins during SSF, which enhanced their solubility in the liquid phase increasing their adsorption and surface activities. This could be occurring in the fermented PPEF samples since solubility decreased over fermentation time in samples and were unable to readily reach the air-water interface making them unstable. However, Elkhalifa *et al.* (2005) found sorghum flour had no FC, and attributed this observation to sorghum flour proteins in solution increasing the surface tension of the water resulting in no formation of foam. A decrease in FC and FS with an increase of fermentation time occurred in fermented pigeon pea seed flour, which was comparable to fermented PPEF (Adebowale & Maliki, 2011). In contrast to fermented PPEF, *A. oryzae* SSF caused an increase in FC and FS of black-eyed pea seed flour with increasing fermentation time (Chawla *et al.*, 2017). This was attributed to fermentation modification that resulted in the significant increase in WHC and electrostatic charge in black-eyed seed flour, which increased FC and FS. A study of three fermented legume varieties: faba bean, desi and kabuli chickpea were found to have a significantly-lower FS than respective raw samples. The author stated that fermented flour samples have different foaming properties depending upon the type of legume, and can be potentially exploited as food ingredients (Chandra-Hioe *et al.*, 2016). Perhaps, this can indicate that foaming properties are highly-dependent on the legume used and cultivation of the legume since properties varied greatly among legumes. Therefore, a decrease in the FC and FS over fermentation time in both PPEF samples could be due to the pea cultivar, environmental factors and plant genetics influencing poor foaming.

Solubility is an important factor that can influence foaming properties. The decrease in solubility over the fermentation time could cause the decrease in PPEF's foaming capacity. Soluble globular proteins are displaced by diffusion at the air-water interface which allows for surface tension reduction. If the proteins are insoluble then their ability to diffuse at the air-water interface decreases, reducing the surface tension and as seen by Elkhalifa *et al.* (2005) no foam can be formed. Fermented PPEF results indicated that foams decreased in FS over the fermentation time for both strains, a finding that may be attributed to weak protein-protein interactions (via electrostatic repulsion, hydrophobic interactions and hydrogen bonds) (Zayas, 1997). Unfolded protein should reorient around the foam and stabilize the bubbles through these interactions because of these weak interactions the bubbles collapse.

(d) Oil and water holding capacities

Oil-holding capacity (OHC) is the amount of oil that can be absorbed per gram of protein. Similarly, water holding capacity (WHC) is the amount of water that can be absorbed per one gram of protein (Boye *et al.*, 2010b). OHC and WHC are two significant functional properties in food application. The OHC and WHC are important parameters in food processing and in formulating a food ingredient since they can influence both storage (e.g., storage conditions and product shelf-life) and food structure (e.g., wet, dry or brittle). The OHC and WHC of fermented PPEF inoculated with fungal strains, *A. oryzae* or *A. niger*, are summarized below in Figures 4.1.5. An interaction effect on OHC between fermentation time and microorganism were found to be statistically significant ($p<0.001$) (Table 4.2). *A. oryzae and A. niger* inoculated PPEF samples were shown to increase the OHC over the duration of fermentation from 1.25 g/g to 1.39 g/g and 1.18 g/g to 2.27 g/g, respectively. The increased OHC is attributed to hydrophobic patch exposure through the action of fungi secreting protease which cleave the protein. It is hypothesized that the hydrophobic patches on the protein enable the proteins to aggregate into a protein cluster. This cluster has space in between the proteins called micro-capillaries which physically-entrap oil. Avramenko *et al.* (2013) reported that limited enzymatic hydrolysis modified the protein, leading to exposed hydrophobic groups that aggregated together via hydrophobic interactions. Thus, short fermentation times may hydrolyze PPEF through the action of fungal protease, exposing the PPEF's hydrophobic interior which then formed a large aggregate (via hydrophobic interactions).

Due to the large aggregate structure, entrapment of oil is a plausible explanation of structure stability.

A similar phenomenon was shown in numerous studies reporting a significant increase of OHC in fermented legumes and cereal flours including: kabuli chickpea, desi chickpea, red bean, faba bean, black-eyed pea, cowpea, sorghum, pearl millet, maize flours and peanut protein concentrate (Yu *et al.*, 2007; Alka *et al.*, 2012; Xiao *et al.*, 2015; Chandra-Hioe *et al.*, 2016; Chawla *et al.*, 2017; Xiao *et al.*, 2018). In fermented black-eyed pea, sorghum and cowpea flours OHC increased from 0.46 g/g (0 h) to 0.91 g/g (96 h), 1.72 g/g (8 h) to 1.80 g/g (24 h), and 0.69 g/g (0 h) to 0.93 g/g (24 h), respectively, over the fermentation time (Prinyawiwatkul *et al.*, 1997; Elkhalifa *et al.*, 2005; Chawla *et al.*, 2017). The OHC increase was attributed to fermentation modification which exposed hydrophobic sites of amino acids present on the surface of the protein that allowed for the unfolding non-polar residues from the interior protein molecules. The surface availability of hydrophobic amino acids could have positively affected the oil binding (Xiao *et al.*, 2015; Chawla *et al.*, 2017; Xiao *et al.*, 2018). Additionally, the hydrocarbon chains of oil are readily available to interact with the hydrophobic groups that are exposed, and thus, are able to hold larger amounts of oil (Xiao *et al.*, 2018).

However, fermentation was shown to decrease OHC in velvet bean (*Mucuna pruriens)* from 2.20 g/g (0 h) to 0.88 g/g (over 72 h) (Udensi & Okoronkwo, 2006). The decline in OHC could be attributed to a greater amount of hydrophilic amino acids exposed through proteolytic activity. Comparing PPEF fermentation using the two fungal strains revealed that *A. niger* had a higher OHC of 2.27 g/g (6 h); whereas, *A. oryzae* inoculated PPEF entrapped almost half as much at 1.39 g/g (6 h). Therefore, *A. niger* would be preferable as an ingredient in certain products due to its increased OHC which, hypothetically, would have an improved mouthfeel and flavour. The increase of OHC could be attributed to its ability to produce more protease over the 6-h, which lead to hydrolyzation of the protein exposing the hydrophobic patches on the protein that were able to fold and re-orientate into an aggregate.

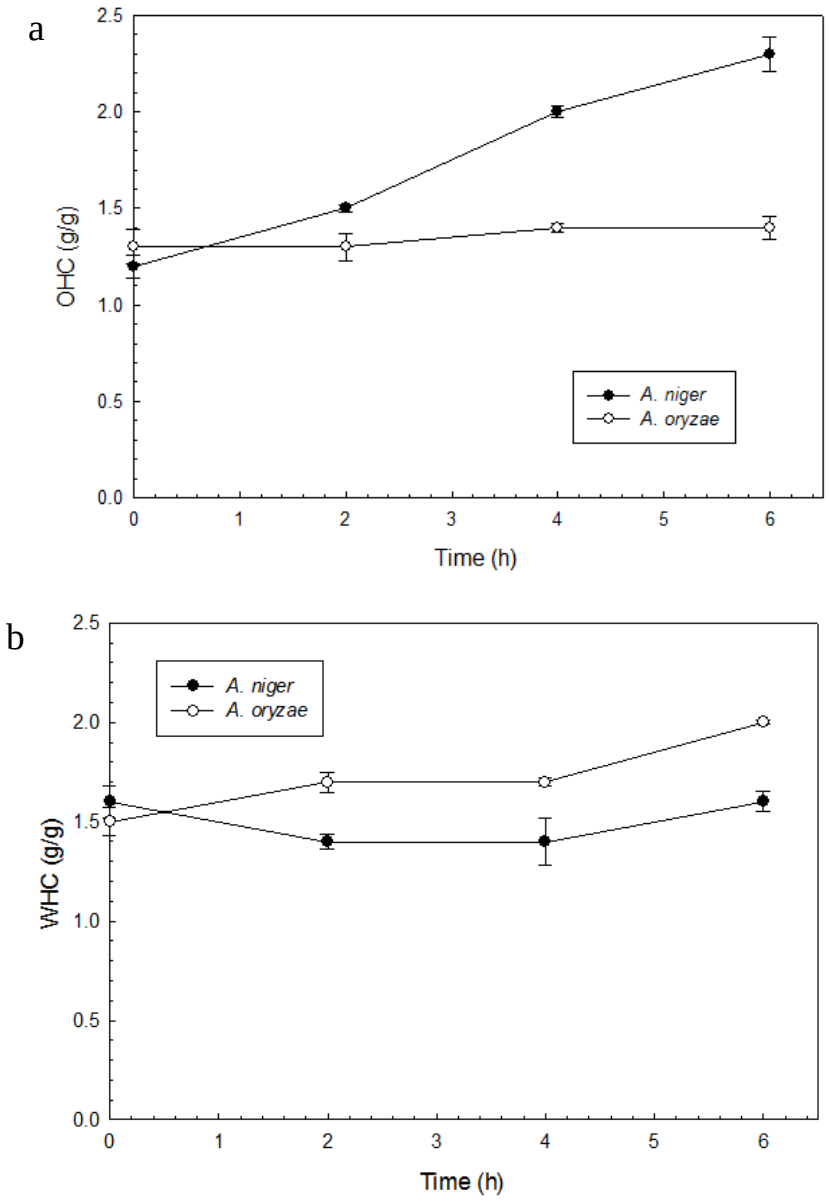

Figure 4.5 (a) Oil holding capacity and (b) water holding capacity of fermented PPEF. Data represent the mean of triplicate measurements on flour ± one standard deviation (n = 3). *Abbreviations:* PPEF (pea protein-enriched flour); OHC (oil holding capacity); WHC (water holding capacity); *A. oryzae (Aspergillus oryzae); A. niger (Aspergillus niger).*

A two-way ANOVA of WHC determined that there was a statistically significant interaction between fermentation time and fungi strain ($p<0.001$). The WHC increased over the duration of fermentation from 1.46 g/g to 2.03 g/g and 1.60 g/g to 1.61 g/g for *A. oryzae* and *A. niger*, respectively. The ability of water holding is dependent upon the availability of hydrophilic groups which bind water molecules. The WHC was shown to increase, a result that could be attributed to the fungus's proteolytic activity cleaving proteins into smaller peptide bonds exposing hydrophilic sites on protein. Most proteins are hydrophilic in nature due to their numerous polar side chains and their peptide back-bone (Prinyawiwatkul *et al.*, 1997). Additionally, the hydrophilic surface formed through hyphae production via filamentous fungi during the fermentation aided in the ability of greater binding of water, and offers a mechanistic explanation. Similar studies have reported the increase of WHC of chickpea, red bean, black-eyed pea, oil bean and drumstick tree (*Moringa oleifera)* flours were improved through fermentation (Akubor & Chukwu, 1999; Reyes-Moreno *et al.*, 2004; Oloyede *et al.*, 2015; Xiao *et al.*, 2015; Chandra-Hioe *et al.*, 2016; Chawla *et al.*, 2017; Xiao *et al.*, 2018). The WHC increase of fermented flours were attributed to fermentation altering the protein confirmation through proteolytic activity. The protease hydrolyzed peptide bonds which increased the number of polar amino acid side chains with strong water holding ability. Furthermore, the increased time of fermentation was shown to influence greater WHC in drumstick tree (*Moringa oleifera)* seed flour ranging from 0.86 g/mL (0 h) to 1.81 g/mL (72 h) (Oloyede *et al.*, 2015). However, Adebowale & Maliki (2011) observed a decrease in WHC of pigeon pea over a 5-day fermentation period from 1.42 g/g to 1.13 g/g. The decrease of WHC hypothetically occurred due to fermentation changing the conformation of protein molecules and exposing fewer hydrophilic groups compared to hydrophobic groups. Comparing the two fungal strains used to inoculate the PPEF, *A. oryzae* resulted in lower OHC and higher WHC than *A. niger* over the 6-h fermentation period. The hydrophilicity of proteins increased during *A. niger* fermentation might be due to the protease activity exposing more hydrophilic groups, whereas in *A. oryzae* more hydrophobic groups were exposed in fermentation. Aggregates could form since the subunit bonds (such as, disulfide bonds, electrostatic repulsion and hydrophobic interactions) are broken through protease activity and when re-dispersed into solution the exposed hydrophobic subunits have a greater affinity for aggregation. Overall, these results suggested that fermented PPEF at 6-h of fermenting had good OHC and WHC.

4.1.5 Conclusion

Study 1 evaluated the physicochemical and functional properties of fermented PPEF inoculated with two different fungi (*A. oryzae* and *A. niger)* over a 6-h fermentation duration. Overall, fermented PPEF altered the protein structure via proteolytic enzymes secreted from the fungi, thus hydrophobic moieties were exposed favoring protein-protein interactions leading to the formation of aggregates. Additionally, fungal fermentation produces hyphae under moist environment conditions, making the substrate hydrophilic in nature. Surface hydrophobicity, zeta potential and nitrogen solubility were all shown to decrease over SSF. The reduction was attributed to proteins having the affinity to aggregate to other proteins exposed through fungal protease. Solubility was affected by the pH; properties were enhanced shifting the pH away from the pI of the protein, thus greater effects were found at pH 3.0 and 7.0. Emulsifying properties remained unchanged and foaming properties were negatively affected by SSF. Whereas, WHC and OHC improved with SSF. *A. oryzae* and *A. niger* used in inoculation were shown to follow similar trends throughout the fermentation and alteration of PPEF. Findings suggested that SSF was effective at modification of PPEF's physicochemical and functional properties. Specifically, OHC and WHC were significantly improved through fermentation, and fermented PPEF could potentially be used as an ingredient in food products such as, baked goods or as a meat binder where improved OHC and WHC is of need.

4.1.6 Connection to study 2

Study one was performed to analyze the proximate content and functionality of fermented PPEF as an ingredient. Protein functionality is highly important when incorporating into a food product to ensure food structure and stability, such as emulsions and foams (Foegeding and Davis, 2011). Since the PPEF was modified via SSF with the intent to improve the functionality, evaluating these affects could indicate whether the modification had a positive or negative outcome and whether fermented PPEF has potential as an ingredient in specific foods. However, study one alone does not fully-indicate the full potential of fermented PPEF as a food ingredient. The modification through fermentation could potentially impact the digestibility and overall quality of the protein. Accordingly, study two focused on the effect of fermented PPEF on bioactive properties, amino acid content and protein digestibility in order to highlight its nutritional significance.

4.2 Effect of fermentation time on the nutritional properties of PPEF fermented by *Aspergillus oryzae* and *Aspergillus niger*

4.2.1 Bioactive compounds

Trypsin is a serine protease enzyme that is a digestive enzyme and has a high specificity to lysine and arginine residues. Trypsin inhibitory activity (TIA) occurs within the protein matrix by formation of inhibitor-trypsin enzyme complex, inhibiting the digestibility of the protein; thus, removal of TIA would increase the nutritional value and digestibility (Shi *et al.*, 2017). Therefore, trypsin inhibitors are also known as an anti-nutritional property since they inhibit digestibility. Fermentation of PPEF was evaluated to determine the levels of TIA over the duration of fermentation (Table 4.9). TIA of fermented PPEF was found to have a statistically significant interaction with fermentation time and fungal strains ($p<0.001$) (Table 4.8). TIA decreased in fermented PPEF over the duration of fermentation by *A. oryzae* and *A. niger* from 28.6 to 19.9 TIU/mg and 25.1 to 22.2 TIU/mg, respectively. During fermentation, microbial activity led to enzymatic hydrolysis enabling the breakdown and degradation of proteinous trypsin inhibitors into smaller units. The degradation of the complex trypsin inhibitors led to simpler and more soluble proteins and thus, lower activity.

Similar to trypsin, chymotrypsin is a serine protease in human digestion system, and cleaves peptide at hydrophobic residues such as tyrosine, tryptophan and phenylalanine, degrading proteins for assimilation. Chymotrypsin inhibitors are small proteins that bind to chymotrypsin enzyme, thereby, limiting protein digestibility. A two-way analysis of variance (ANOVA) found that chymotrypsin inhibitory activity (CIA) had a statistically-significant interaction between fermentation time and fungi ($p<0.001$) (Table 4.8). CIA decreased over the 6-h fermentation time from 76.3 to 59.7 CIU/mg and 80.2 to 61.9 CIU/mg for *A. oryzae*- and *A. niger*-fermented PPEF samples, respectively (Table 4.9). SSF hydrolyzes peptide into free amino acids through microbial activity, thus CIA is hypothesized to be reduced through cleavage of the chymotrypsin inhibitory peptide.

The presence of inhibitors in legumes has been demonstrated to reduce digestibility to varying degrees, dependent on the processing method utilized, and properties exhibited by the type of legume.

Table 4.8 Two-way analysis of variance (ANOVA) analysis of fermented PPEF bioactive properties and protein quality.

	Bioactive properties			Protein quality	
	TIA	**CIA**	**TPC**	**IVPD**	**IV-PDCAAS**
Main effects					
Fungi	p<0.001	p<0.001	p<0.001	p<0.001	NS
Time	p<0.001	p<0.001	p<0.001	p=0.001	p<0.001
Interactions					
Fungi × time	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001

Conditions:
Fungi (*Aspergillus oryzae* and *Aspergillus niger)*
Time (0, 2, 4, and 6 h)
All measurements were performed in triplicate on the composite blend from three fermented batches. Data represent the mean ± one standard deviation (n = 3).
Abbreviations: PPEF (pea protein-enriched flour); TIA (trypsin inhibitory activity); CIA (chymotrypsin inhibitory activity); TPC (total phenolic content); IVPD (*in vitro* protein digestibility); IV-PDCAAS (*in vitro* protein digestibility corrected amino acid score) and NS (not significant).

Table 4.9 Bioactive properties of fermented PPEF.

Sample	TIA (TIU/mg)	CIA (CIU/mg)	TPC (mg GAE/100g)
A. oryzae			
0 h	28.57±0.80	76.26±0.46	3.83±0.19
2 h	21.32±0.50	73.54±0.51	4.48±0.12
4 h	19.45±0.09	60.22±0.68	5.44±0.02
6 h	19.89±0.45	59.72±0.34	5.51±0.01
A. niger			
0 h	25.07±0.43	80.23±0.70	3.38±0.09
2 h	24.06±0.75	73.55±0.44	3.59±0.03
4 h	22.73±0.85	62.89±0.79	3.70±0.04
6 h	22.19±0.65	61.89±0.70	4.65±0.31

All measurements were performed in triplicate on the composite blend from three fermented batches. Data represent the mean ± one standard deviation (n=3).
Abbreviations: PPEF (pea protein-enriched flour); TIA (trypsin inhibitory activity); CIA (chymotrypsin inhibitory activity); TPC (total phenolic content); A. *oryzae* (*Aspergillus oryzae*) and *A. niger* (*Aspergillus niger*); GAE (Gallic acid equivalent).

Thus, reduction of TIA has been demonstrated through fermentation of *Vigna racemose* flour, dry beans and soybean meal inoculated with *Rhizopus oligosporus, A. niger, Lactobacillus fermentum, A.oryzae and Lactobacillus brevis*, respectively (Baramapama & Simard, 1994; Reddy & Pierson, 1994; Granito *et al.*, 2002; Gao *et al.*, 2013; Difo *et al.*, 2015). Additionally, natural fermentation has been demonstrated to decrease the TIA of flour and whole bean seeds (*Phaseolusvulgaris)* (Granito *et al.*, 2002). Gao *et al.* (2013) found SSF to be an effective process to reduce TIA in soybean meal when inoculated with *Lactrobacillus brevis* or *A. oryzae,* showing a 57.1% and 89.2% reduction, respectively. The decline in TIA was attributed to microorganism's hydrolysis of the protein during the fermentation process. In comparison, TIA of PPEF underwent a 30.4% and 11.5% reduction when inoculated with *A. oryzae* and *A. niger,* respectively. Therefore, reduction of TIA via fermentation may be useful to improve the nutritional quality in PPEF with respect to protein digestibility. CIA showed a similar trend to TIA, and thus decreased over fermentation time in PPEF samples by 21.8% and 22.8% for *A. oryzae* and *A. niger*, respectively. Both chymotrypsin and trypsin inhibitors are low molecular weight proteins, making them heat-labile. Thermal treatment affects the intermolecular bonds holding the inhibitors tertiary structure, this causes a break and change to the active site conformation (Aviles-Gaxiola *et al.*, 2018). Thermal treatments have been extensively studied to degrade inhibitors in legumes. Specifically, in pea seeds thermal treatments were used to reduce trypsin inhibitors finding that boiling (100ºC for 20 min) and pressure cooking (120ºC for 10 min) inactivated TIA completely (Aviles-Gaxiola *et al.*, 2018). Generally, using a high temperature for a short period of time or a lower temperature over a longer period of time was shown to reduce inhibitors. Ma *et al.* (2017) found that the low molecular weights of trypsin and chymotrypsin inhibitors can be easily targeted through denaturation from the heat used during fermentation. Gao *et al.* (2013) found the degradation of trypsin inhibitors through fermentation processing with *A. oryzae* and *Lactobacillus plantarum*, determining that at 37ºC for 5 days decreased the TIA in soybean meal by 89.2% and 99.2%, respectively. In this study, SSF of PPEF was carried out for 6-h at 40ºC with a reduction in TIA and CIA. When compared to the 5-d fermented soybean meal study by Gao *et al.* (2013), the reduction values of PPEF fermented for 6 h were not as significant. This could be due to the shorter fermentation period which was unable have as a significant effect via thermal treatment on the trypsin and chymotrypsin inhibitors. However, thermal treatment could have a slight effect on reducing TIA

and CIA in fermented PPEF. Overall, the decline is mainly attributed to the microorganism's hydrolysis of the protein into simpler complexes.

The total phenolic content (TPC) in plants is related to chemical structures that are conjugated through hydroxyl groups with sugar as glycosides (Lin *et al.*, 2006). Phenolic compounds are known to be health-beneficial due to their antioxidant activity. The interaction between fermentation time and fungal strain was found to have a significant effect on TPC ($p<0.001$) (Table 4.8). Gallic acid is used as the standard in determination of phenolic content by Folin-Ciocalteau assay, wherein results are reported in gallic acid equivalents (GAE). TPC increased throughout the fermentation time course, ranging from 3.8 to 5.5 mg GAE/100g and 3.4 to 4.7 mg GAE/100g for *A. oryzae* and *A. niger* PPEF samples, respectively (Table 4.9). The increase in TPC in fermented PPEF could be attributed to polyphenols cleavage from protein complexes through proteolytic activity, i.e., releasing polyphenols. This could potentially explain how TPC increases in fermented PPEF. In literature, fermentation has been reported to increase the content of bioactive phenolic compounds in legumes and furthermore enhance their antioxidant activity. TPC was reported to increase in lentil and soybean through SSF processing (Lin *et al.*, 2006; Fernandez-Orozco *et al.*, 2007; Torino *et al.*, 2013). Lentil SSF inoculated with *B. subtilis* resulted in a significant increase of 24 mg GAE/g after 48 h of fermentation and increased further after 96 h to 34-35 mg GAE/g (Torino, *et al.*, 2013). This late-phase fermentation increase could be due to lentil's different composition of the proximate constituents (i.e., protein, carbohydrate, lipid and ash) when compared to pea. However, the TPC trend was similar to PPEF, which also increased over fermentation time. Fernandez-Orozco *et al.* (2007) observed an increase in phenolic content after fermentation with *A. oryzae, Rhizopus oryzae* and *Bacillus subtilis* of soybean. This was attributed to fermentation hydrolyzing complexes of polyphenols into simpler ones. Additionally, Duenas *et al.* (2005) stated the complex polyphenols hydrolyzed during SSF resulted in simpler and superior biological active compounds. Schmidt *et al.* (2014) reported an increase in free phenolic content through SSF by filamentous fungi's production of enzymes, that was attributed to cleavage of compounds complexed with lignin. Phenolics are generally present in chain formation and linked components such as, proteins, cellulose and lignin are attached through ester linkages. Therefore, when cleavage occurs through hydrolysis of microbial enzymes, phenolic content was shown to increase (Schmidt *et al.*, 2014). PPEF composition is presumed to contain approximately ~45% of carbohydrate. Thus, complexes of carbohydrates and proteins

attached to phenolics potentially could be cleaved during SSF increasing the total phenolic content. The increase in phenolic content through fermentation has been demonstrated to improve their health-linked functionality due to conjugate forms of phenolic compounds becoming bioconverted into their free forms (Torino *et al.*, 2013). Therefore, the overall quality of PPEF can be improved if TPC are hydrolyzed into their free conjugate forms during SSF.

4.2.2 Protein quality

Protein quality is an important criterion for adequate nutrition and maintenance of good health. A major drawback of plant pulse crops like pea is their low digestibility of protein due to their high presence of bioactive compounds. The protein digestibility of pulses could be greatly influenced by globular structure, conformation of protein and bioactive compounds that can inhibit digestive enzymes. Processing through fermentation of PPEF provides an opportunity to increase protein digestibility and amino acid availability. Protein quality of PPEF as a function of fungal strain and fermentation time is given in Table 4.8. The *in vitro* protein digestibility (IVPD) measures the change in pH drop which results from the release of amino acids and peptides, as the proteins are digested (Tinus *et al.*, 2012). The IVPD of fermented PPEF was found to increase over fermentation time 74.77% to 80.92% and 74.89% to 79.36% for *A. oryzae* and *A. niger* samples, respectively (Table 4.10). The IVPD was found to have a statistically-significant interaction between fermentation time and fungal strains ($p<0.001$) (Table 4.8). During SSF, the production of fungal proteases hydrolyzed the peptide chains into amino acids, cleaving and reducing the anti-nutritional factors, such as trypsin and chymotrypsin inhibitors, and in turn making the fermented PPEF more digestible over time. Fermentation was reported in previous studies to significantly improve the IVPD for cereal and legume samples (Yousif & Tinay, 2001; Granito *et al.*, 2002; Angulo-Bejarano *et al.*, 2008; Alka *et al.*, 2012; Chandra-Hioe *et al.*, 2016; Xiao *et al.*, 2018). Chandra-Hioe et al (2016) found a similar trend in chickpea and faba bean flours to fermented PPEF examined in this thesis research, with IVPD increasing as fermentation time increased. This effect is assumed to be due to the increase in protease activity and decrease in anti-nutritional factors over fermentation time. The decrease in anti-nutritional factors was also demonstrated in the PPEF samples (Table 4.9).

Angulo-Bejarano *et al.* (2008) found IVPD was improved by SSF; proteins from tempeh flour resulted in an IVPD of 83.20%.

Table 4.10 Amino acid scores and protein data of fermented PPEF.

Sample	Limiting amino acid[1]	Limiting amino acid score[1]	IVPD[2] (%)	IV-PDCAAS [1] (%)
A. oryzae				
0 h	MET + CYS	0.77	74.77±0.55	66.68±0.32
2 h	MET + CYS	0.76	71.11±0.69	65.02±0.12
4 h	MET + CYS	0.74	79.78±0.65	63.26±0.29
6 h	MET + CYS	0.74	80.92±0.42	63.50±0.04
A. niger				
0 h	MET + CYS	0.80	74.89±0.79	69.33±0.07
2 h	MET + CYS	0.79	74.17±0.36	68.74±0.18
4 h	MET + CYS	0.72	75.43±0.31	61.10±0.11
6 h	MET + CYS	0.69	79.36±0.91	59.00±0.11

[1]Measurements were performed once on one sample from the triplicate batches.
Measurements were calculated by using amino acid content in 1 g of test protein/the same amino acid content in 1 g of reference protein determined by FAO.
[2]Measurements were performed in triplicate on the composite blend from three batches of fermentation. Data represent the mean ± one standard deviation.
Abbreviations: MET (methionine); CYS (cysteine); IVPD (*In vitro* protein digestibility); IV-PDCAAS (*In vitro* protein digestibility corrected amino acid score); *A. oryzae* (*Aspergillus oryzae)*; *A. niger* (*Aspergillus niger);* and PPEF (pea protein-enriched flour).

The authors attributed this to the elimination of anti-nutritional factors through hydrolytic reactions. The decrease in anti-nutritional factors allowed for the protein cross-linking to be more susceptible to proteolytic attack, thereby increasing digestibility. Furthermore, other studies showing increases in IVPD of fermented products were presumably due to the opening of proteins during SSF through protease activity, which allowed for partial degradation of complex storage proteins into more simple, stable and soluble proteins (Yousif & Tinay, 2001; Granito *et al.*, 2002; Alka *et al.*, 2012; Fawale *et al.*, 2017). Alka *et al.* (2012) reported the effect of pH reduction during the fermentation as having enhanced proteolytic enzyme activity; this occurs through, the breakdown of proteins

into smaller polypeptides which are then easily digested by enzymes. In comparison, the decrease in pH occurred in the PPEF samples as shown in Figure 4.1. Thus, pH reduction during fermentation could be a factor influencing the digestibility. A low IVPD could also be due to the presence of anti-nutrients. For example, binding of trypsin inhibitors to the endopeptidase trypsin to form an inactive protein complex, thus inhibiting the activity of trypsin and decrease the protein digestibility. Additionally, resistance of globulins to proteolytic enzymes would also result in a reduction of IVPD (Xiao *et al.*, 2018). An increase in IVPD could also be greatly influenced by the protocol used to determine IVPD in the fermented PPEF, which has been criticized (Tinus *et al.*, 2012). Since the equation: IVPD (%) $= 65.66 + 18.10 * \Delta pH_{10\,min}$ (Eq. 13), the IVPD has a minimum of 65.66%, even if no change in pH occurs over the allotted time in the experiment. Additionally, a value greater than 100% could be obtained depending on the magnitude of pH change (Tinus *et al.*, 2012). However, an underestimation of human digestibility could also occur since the small intestine enzymes are only being mimicked. Potentially more enzymes could be involved and digest a greater amount of the protein than seen in this IVPD analysis.

In vitro protein digestibility amino acid score (IV-PDCAAS) was used to determine the digestibility while taking amino acid score into account. The reduction of anti-nutritional factors and the alteration of amino acid profile of a protein source through fermentation would alter the IV-PDCAAS. PDCAAS is the main test used by the Food and Agriculture Organization of the United Nations (FAO) for accessing global food protein quality since it incorporates essential amino acids of food protein and digestibility is an accurate analysis. Complete proteins have PDCAAS score of 1.00, which can lead to an underestimation of protein quality if the protein score is higher than 1.00 (Nosworthy *et al.*, 2017a). However, an overestimation may occur since digestion of protein may occur from bacteria in the colon after passing through the intestine. *In vitro* was selected as a test of digestibility over *in vivo* due to its feasibility, simplicity, reproducibility, quick analysis and lower cost. Whereas, *in vivo* is time consuming, expensive and requires specialized equipment; thus, it is not feasible for every lab to employ (Urbano *et al.*, 2005). Additionally, Nosworthy *et al.* (2018a) stated that the IV-PDCAAS method could potentially be used in the future as a surrogate for *in vivo* evaluation of pulse protein ingredients.

IV-PDCAAS of PPEF was found to decrease over the fermentation time from 66.68% to 63.50% and 69.33% to 59.00% for *A. oryzae* and *A. niger*, respectively (Table 4.10). The IV-PDCAAS was found to have a statistically-significant interaction between fermentation time and

fungal strain on fermented PPEF ($p<0.001$) (Table 4.8). The reduction in IV-PDCAAS scores in the fermented PPEF reflect the reduction in the concentration of the limiting amino acid. The full amino acid composition of fermented PPEF, reported in grams per 100 g of flour, is given in Table 4.11. The limiting amino acids of fermented PPEF throughout fermentation remained methionine and cysteine. The limiting amino acid values ranged from 0.77 to 0.74, *A. oryzae* and 0.80 to 0.69, *A. niger* in fermented PPEF. The limiting amino acid score for fermented PPEF is consistent with literature since legumes are known to be limiting in sulfur amino acids and tryptophan (GL-Pro, 2005; Nosworthy *et al.*, 2018a). The decrease over fermentation time could be due to the fungi utilizing these amino acids further reducing the essential amino acids. Overall, PPEF decreased in PDCAAS values over the fermentation time, which occurred due to the limiting amino acids decrease. Nosworthy *et al.* (2018a) determined the PDCAAS of red and green lentils when processed through various methods such as extrusion, cooking and baking, similarly methionine and cysteine to be the limiting amino acids. The lentils ranged from 0.57 for baked green lentil, to 0.68 for extruded red lentils. Additionally, tryptophan was also found to be limiting in the processed lentil samples ranging from 0.69 for extruded red lentil, to 0.78 for extruded green lentils. The fermented PPEF samples were shown to be slightly higher in the limiting amino acid score than the lentil samples which would be expected since they are different species. Nosworthy *et al.* (2018b) IV-PDCAAS results ranged from a low value of 49.81% for faba bean to 70.19% for pinto bean, which were both processed through cooking. Whereas, yellow pea ranged from 62.27% using extrusion to 67.44% for cooking (Nosworthy *et al.*, 2017b). The fermented PPEF values were within range of these two studies, ranging from the highest value of 69.33% for *A. niger* (0 h) to the lowest value of 59.00% for *A. niger* (6 h). Nosworthy *et al.* (2017b; 2018b) indicated that processing method strongly determined the optimum protein quality. Fermentation was an effective processing method to increase digestibility; however, the limiting amino acids were decreased (via fungi utilization) affecting the overall IV-PDCAAS score.

Table 4.11 Amino acid composition (g per 100 g of flour, on an *as is* basis) for fermented PPEF.

Sample	***A. oryzae***				***A. niger***			
Time	**0 h**	**2 h**	**4 h**	**6 h**	**0 h**	**2 h**	**4 h**	**6 h**
Amino Acid								
Aspartic Acid	4.61	4.97	4.64	4.89	4.60	4.55	4.45	4.50
Glutamic Acid	6.62	7.28	6.86	7.12	6.80	6.57	6.52	6.64
Serine	1.92	2.09	2.00	2.08	2.00	1.97	1.92	1.89
Glycine	1.39	1.59	1.52	1.55	1.45	1.40	1.41	1.45
Histidine‡	0.86	0.96	0.90	0.91	0.88	0.84	0.79	0.85
Arginine	3.37	3.44	3.42	3.50	3.40	3.33	3.19	3.36
Threonine‡	1.44	1.59	1.51	1.53	1.48	1.43	1.41	1.44
Alanine	1.44	1.59	1.49	1.56	1.46	1.43	1.40	1.44
Proline	1.62	1.79	1.73	1.77	1.66	1.64	1.61	1.62
Tyrosine	1.39	1.60	1.43	1.39	1.40	1.35	1.39	1.45
Valine‡	1.42	1.64	1.55	1.46	1.46	1.37	1.37	1.60
Methionine*‡	0.44	0.53	0.44	0.45	0.45	0.45	0.46	0.46
Cysteine*	0.47	0.56	0.46	0.46	0.48	0.47	0.46	0.46
Isoleucine‡	1.41	1.60	1.50	1.43	1.41	1.34	1.32	1.58
Leucine‡	2.78	3.10	2.93	2.97	2.85	2.77	2.76	2.80
Phenylalanine‡	1.95	2.20	2.08	2.09	1.98	1.91	1.91	1.99
Lysine‡	2.83	2.96	2.74	2.96	2.72	2.77	2.60	2.81
Tryptophan‡	0.45	0.46	0.45	0.43	0.45	0.45	0.42	0.44

Measurements were performed once on each flour sample.
(*) Indicates sulfur amino acid. (‡) Indicates essential amino acids.
Abbreviations: PPEF (Pea protein-enriched flour); *A. oryzae* (*Aspergillus oryzae*)*; A.niger* (*Aspergillus niger*).

Table 4.12 Essential amino acid concentration (mg/g protein) for fermented PPEF.

	Amino acids								
Sample	**THR**	**VAL**	**MET + CYS**	**ILE**	**LEU**	**PHE + TYR**	**HIS**	**LYS**	**TRP**
A. oryzae									
0 h	31	30	19	30	59	71	18	61	10
2 h	32	32	19	31	59	73	19	56	9
4 h	31	32	19	31	60	72	18	56	9
6 h	31	30	18	29	60	71	18	60	9
A. niger									
0 h	32	31	20	30	61	73	19	59	10
2 h	31	30	20	29	60	71	18	60	10
4 h	28	27	18	26	54	65	16	51	8
6 h	27	30	17	30	52	64	16	53	8
FAO reference	34	35	25	28	66	63	19	58	11

Measurements were performed once on each flour sample.
Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); and TRP (tryptophan); *A. oryzae (Aspergillus oryzae); A. niger (Aspergillus niger);* and PPEF (pea protein enhanced flour).

Table 4.13 Amino acid scores for fermented PPEF.

	Amino acids								
Sample	**THR**	**VAL**	**MET + CYS**	**ILE**	**LEU**	**PHE + TYR**	**HIS**	**LYS**	**TRP**
A. oryzae									
0 h	0.90	0.87	*0.77	1.08	0.90	1.13	0.96	1.04	0.88
2 h	0.96	0.93	*0.76	1.12	0.89	1.16	1.01	0.97	0.83
4 h	0.91	0.90	*0.74	1.10	0.91	1.14	0.97	0.97	0.83
6 h	0.92	0.85	*0.74	1.04	0.92	1.12	0.08	1.04	0.80
A. niger									
0 h	0.94	0.90	*0.80	1.08	0.93	1.15	0.99	1.01	0.88
2 h	0.91	0.85	*0.79	1.03	0.91	1.12	0.96	1.03	0.88
4 h	0.81	0.77	*0.72	0.92	0.82	1.03	0.82	0.88	0.75
6 h	0.79	0.86	*0.69	1.06	0.79	1.02	0.84	0.91	0.76

Measurements were performed once on each flour sample.
(*) Indicates the first limiting amino acid.
Abbreviations: THR (threonine); CYS (cysteine); VAL (valine); MET (methionine); ILE (isoleucine); LEU (leucine); TYR (tyrosine); PHE (phenylalanine); HIS (histidine); LYS (lysine); TRP (tryptophan); *A. oryzae (Aspergillus oryzae); A. niger (Aspergillus niger);* and PPEF (pea protein enhanced flour).

4.2.3 Conclusion

Overall, the trypsin and chymotrypsin inhibitory activities decreased over the duration of fermentation; whereas, total phenolic content increased. This was hypothesized to occur due to the production of enzymes during fermentation, in the case of trypsin and chymotrypsin inhibitory activities, the proteinous inhibitors were hydrolyzed and reduced into soluble proteins. Meanwhile, the increase of total phenolic content resulted from the fermentation hydrolyzing complexes of polyphenols into simpler ones. The protein quality (IVPD) increased which was attributed to the reduction of trypsin and chymotrypsin inhibitory activities. Whereas, the IV-PDCAAS decreased potentially due to the decrease in the limiting amino acids methionine and cysteine. Since SSF with *A. niger* and A. *oryzae* led to poor protein quality, it is not recommended as a means for altering the nutritional value of pea protein enriched flour.

5. OVERALL CONCLUSIONS

Pea protein is a highly available plant crop grown globally that contains high-quality protein composition and is rich in essential amino acids. The potential for incorporation of pea protein as an ingredient into the human diet could have health benefits (e.g., high protein quality and total phenolics), as well as sustainable and economical value. However, pulse crops have limitations that include the presence of anti-nutritional factors (i.e., trypsin and chymotrypsin inhibitors) which ultimately inhibit complete digestibility of proteins. Biotechnological processing, such as SSF, is a simple, cost-effective and efficient process to increase protein digestibility in pulse ingredients. With the reduction of bioactive compound activities, the increase in digestibility and phenolics and thus, protein quality, functionality of the protein may also be altered. The alteration of functionality, including solubility, emulsification, foaming and water/oil holding capacities, could negatively or positively impact the ingredient, making incorporation either beneficial or impractical. In order to successfully implement a new food ingredient, it is imperative to determine its functional properties for food application. Therefore, this study focused on both factors: functionality and protein quality of PPEF modified through SSF. Overall, this study evaluated how SSF modification impacted the physicochemical, functional and protein quality of PPEF.

In the first study, physicochemical and functional properties of fermented PPEF inoculated with two different fungi (*A. oryzae* and *A. niger)* over a 6-h fermentation was evaluated. Overall, fermented PPEF altered the protein structure via proteolytic enzymes secreted from the fungi, hydrophobic moieties were exposed favoring protein-protein interactions leading to the formation of aggregates. The ratio between hydrophilic and hydrophobic moieties or groups can affect the functionality properties. For example, if there are more hydrophobic groups after hydrolysis through proteolytic activity, then when the protein is re-dispersed into solution the protein would aggregate together. This would ultimately lead to insolubility and affect other major functionality properties. The hydrophilic/hydrophobic ratio is greatly dependent on plant species, environmental factors and genetics (Tzitzikas *et al.*, 2006).

Surface hydrophobicity, zeta potential and nitrogen solubility results showed decreases

over the duration of fermentation due to protein-protein interactions being favored, leading to the formation of large aggregate structures in solution. Solubility was affected by the pH; properties were enhanced upon shifting the pH away from the pI of the protein, thus greater effects were found at pH 3.0 and 7.0. The WHC and OHC were improved with fermentation; whereas, the emulsifying properties remained unchanged and foaming properties were negatively-affected. The fungi used in inoculation were shown to follow similar trends throughout the fermentation and alteration of PPEF. Findings suggested that SSF was effective at modification of PPEF's physicochemical and functional properties. Additionally, fermented PPEF application would be applicable to food products with the need for improved oil/water holding capacities.

The second study evaluated the protein quality of fermented PPEF through analysis of bioactive properties, IVPD and IV-PDCAAS. Overall, trypsin and chymotrypsin inhibitory activities decreased over the duration of fermentation; whereas, total phenolic content increased. This occurred due to the production of enzymes during fermentation; in the case of trypsin and chymotrypsin, they were hydrolyzed and reduced into soluble proteins. Whereas, hydrolyzation (via proteolytic activity) of polyphenols into smaller complexes contributed to an increase in total phenolic content. The protein quality (IVPD) increased which was attributed to the reduction of trypsin and chymotrypsin inhibitory activities. The increase of total phenolic content did not interfere with the increase in IVPD, which could indicate that they were potentially health-beneficial instead of inhibitory to digestion. Whereas, the IV-PDCAAS decreased potentially due to a decrease in the limiting amino acids, methionine and cysteine, resulting from fermentation. Since SSF with *A. niger* and A. *oryzae* led to poor protein quality, it is not recommended as a means for altering the nutritional value of pea protein enriched flour.

6. FUTURE STUDIES

The results of this thesis research demonstrated that SSF had an impact on both protein functionality and quality. Building upon this study could further explain the effect of SSF modification of PPEF and provide insights into what is occurring over the course of fermentation (e.g., hyphae development, enzyme production and site-specific hydrolysis). This study focused solely on *Aspergillus* species, thus further studies could evaluate different fungal genera. Since microorganisms are capable of producing different products, such as enzymes and bioactive compounds, to enhance the food product perhaps another genus would produce a greater amount of enzymes that could hydrolyze the protein more efficiently or cleave at different sites. As mentioned, the hydrolyzation of 7*S* over 11*S* greatly affected the functionality; thus, an alteration of the 7*S* and 11*S* ratio could result in different functionality properties of PPEF.

The number of different enzymes involved in the fungal fermentation process was not documented in this project. An analysis of the protease activity would be an effective analysis to give greater insight into the hydrolysis of the protein throughout fermentation, including how much protease is needed to significantly alter the protein. This could lead to SSF being greater in efficiency because the amount of protease needed to obtain acquired degree of hydrolysis would be known in addition to the time to produce the amount of protease. Furthermore, assessment through scanning electron microscopy of the hyphae growth on the PPEF substrate would provide greater understanding into the hydrophilic and hydrophobic properties. The hydrophobic and hydrophilic properties of the hyphae are presumed to have affected the functional and physiochemical properties of the fermented PPEF, and thus would be beneficial to explore the growth of hyphae over the course of fermentation.

Additionally, more in-depth nutritional value information, such as phytic acid and saponins, could be evaluated throughout fermentation to better understand PPEF as an ingredient. Assessment of these properties could also further explain the improvement in protein quality that was observed in this study. Assessment of carbohydrates and starch digestibility could further improve knowledge of the health benefits of PPEF. Since pulses are generally good sources of slow-release carbohydrates and pea contains a large amount of starch, this may be a future study

to enhance PPEF as an ingredient. Furthermore, evaluation of angiotensin I-converting enzyme inhibitors (ACE inhibitors) activity could enhance understanding of the nutritional value of PPEF as an ingredient. ACE inhibitors are bioactive peptides that can prevent high blood pressure and can potentially improve cardiovascular health. Since ACE inhibitory peptides can be produced through SSF with a legume substrate, perhaps this could be beneficial for control of hypertension (Xiao *et al.*, 2018). The production of ACE inhibitory peptides is through hydrolyzation of legume proteins and since protein hydrolysis occurs throughout the fermentation process, bioactive peptides with ACE inhibitory activity may be liberated and aid in the reduction of hypertension. ACE inhibitory activity potentially could be a value-added ingredient that would benefit the utilization of PPEF.

SSF is a traditional processing method that has been exploited greatly; however, on a larger scale production can be unpredictable. Thus, understanding the limitations of process scale-up would help to ensure that a reproducible, consistent fermented PPEF product is plausible. A sensory evaluation of color, flavour and texture of fermented PPEF should be performed in the future since utilization as a food ingredient is the aim of the final product. Various studies acknowledge that SSF is able to enhance the flavour and reduce the bitterness and beany flavour of pea; therefore, having a sensory panel evaluate this product could help determine if use of fermented PPEF is a viable approach. It is notable that even though a food product with high functionality, nutritional value and digestibility could be produced, consumer acceptability is ultimately the key determinant of whether a product will succeed.

Standardization of methods measuring protein functionality, including water holding capacity, oil holding capacity, emulsification and foaming would be extremely beneficial since the results of each study varies depending on the method used. This makes it difficult to compare results and benchmark further research. If there was a standardized method formulated based on the type of protein (flour, concentrate or isolate) the analysis could be performed, and comparison of results would be easily obtained.

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