# Reducing off-flavour in Plant Protein Isolates by Lactic Acid Fermentation

by

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis entitled:

**Removing Volatile Compound in Plant Protein Isolates by Lactic Acid Fermentation**

Submitted by Yuan Shi in partial fulfillment of the requirement for

the degree of Master of Science

in Food Science

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## Abstract

Pea is a nutritious legume that can be used as an animal protein substitute. However, the unpleasant greeny aroma severely inhibits the application of pea-derived proteins as a food ingredient. This study aims at improving the aroma of pea protein isolates (PPIs) with an aim to employ these PPIs for plant-based dairy substitute production. Major objective of this study is to establish the applicability of lactic acid fermentation (LAF) in eliminating unpleasant aroma from PPIs while maintaining the functional properties and protein quality. We hypothesize that LAF treatment can reduce the undesirable aroma of pea protein isolates by virtue of desirable bacterial action on plant proteins.

A solid-phase microextraction followed by gas chromatography-mass spectrometry (SPME-GC-MS) method was developed in this study to identify and quantify the volatile compound profile of plant-based protein. Total concentration of volatile compounds belonging to aldehyde, ketone and alcohol group in the pea, soy and brown rice protein is analyzed and compared. Different LAF treatments are performed with *Lactobacillus plantarum, Lactobacillus casei* and mixed strains of probiotics. The protein quality of treated and untreated protein sample is analyzed by the Bradford protein assay and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Evolution of functional properties including emulsifying properties, foaming properties, water holding and oil binding capacities of samples with different times of lactic acid fermentation treatments are also analyzed.

Based on the experimental results, the water-soluble protein content decreased with the increase of fermentation time, with protein configuration majorly altered beyond 15-hour of fermentation. Thus, a 10-hour *L. plantarum* fermentation was found optimal in eliminating off-flavour while maintaining protein quality and functional properties. For the optimal treatment,

around 42% aldehyde and 64% ketone content were removed, and a small amount of alcohol was produced. This change of the aroma compound profile is considered desirable for dairy substitute production. Results from a descriptive analysis sensory test showed that LAF treatment successfully decreased the overall aroma and flavour intensity in PPIs. The results of this study could be used by the Canadian Food Industry for pre-treating pea protein isolates, before using them as an ingredient in plant-based dairy substitutes.

## Lay Summary

21st century consumers are increasingly concerned by the sustainability aspects of animal-based products and aware of the associated allergy of dairy products, which has led to the creation of a niche plant-based dairy alternative market. Plant-based proteins are used in such alternatives to provide the necessary protein content, but they often impart a greeny off-flavour to the commercial non-dairy product. The objective of this study is to identify the functionality of lactic acid fermentation (LAF) in improving the taste of pea protein isolates (PPIs), which are to be used downstream for non-dairy substitute production. Results show that LAF was effective in improving the aroma of PPIs, and this method has a huge potential to be used in different plant-based food production. This research will help improve the production and availability of non-allergenic healthy plant-based dairy substitutes in Canadian and global markets.

## Preface

Research conducted in Chapter 3 has been submitted for publication. Singh, A., Shi, Y., Magreault, P., Kitts, D.D., & Singh, A.P. (2020). Analysis of volatile aroma profiles derived from pea, soy and rice protein isolates. The experiment method was designed by Dr. Anika Singh and Dr. Anubhav Pratap Singh, the experiment was performed by me, under the guidance of Dr. Anubhav Pratap Singh and Lina Madilao. Anika Singh and I finished the data analysis and manuscript writing.

Part of the literature review in Section 1.3 has been submitted for publications. Shi, Y., Mandal, R., Singh, A.P. (2020). Legume Lipoxygenase: Strategies for application in food industry. I did all the literature research work. The manuscript was written by me. Ronit Mandal and Dr. Anubhav Pratap Singh reviewed and edited my manuscript.

The study in Chapter 5 and 6 has been submitted to the for publications. Shi, Y., & Singh, A.P. (2020). Lactic acid fermentation: A novel approach to eliminate unpleasant aroma in pea protein isolates. I conducted all the testing and wrote all manuscript. Dr. Anubhav Pratap Singh reviewed and edited my manuscript.

The rest of this work was designed and performed solely by the author Yuan Shi, under the guidance of Dr. Anubhav Pratap Singh.

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## List of Abbreviation

| | |
|---|---|
| ANOVA | Analysis of variance |
| DDMP | 2,3-Dihydro-2,5-dihydroxy-6-methyl-4 H -pyran-4-one |
| DSI | Direct steam injection |
| GC | Gas chromatography |
| GRAS | Generally recognized as safe |
| HPO | Hydroperoxy fatty acids |
| LAB | Lactic acid bacteria |
| LAF | Lactic acid fermentation |
| LOX | Lipoxygenase |
| LSD | Least significant difference |
| MS | Mass spectrometry |
| OAV | Odour activity values |
| POD | Peroxidase |
| PPIs | Pea protein isolates |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SPME | Solid-phase microextraction |

| | |
|---|---|
| SPME-GC-MS | solid-phase microextraction followed by gas chromatography-mass spectrometry |
| $t_R$ | Retention time |
| UBC | University of British Columbia |
| VAW | Volume after whipping |
| VAS | Volume after standing |
| VMD | Vacuum microwave dehydration |

## Acknowledgements

This thesis becomes a reality with the kind support of many individuals. I would like to owe my sincere thanks to all of them.

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I also thank everyone in the UBC Food Processing Engineering Laboratory for creating the most friendly and positive atmosphere in the lab. I appreciate all the valuable suggestions from my lab members before every presentation. Thank my lab members' for sharing their experiences and providing support and help in my experiment and study.

Also, thank you to Lina Madilao and Peter Hoffman for their technical support. Thanks to Lina for teaching me to analyze the data and giving me a lot of professional advice. Thanks to Peter for helping me with all the equipment.

## Dedication

This work is dedicated to my family for their unconditional support and encouragement over the years.

# Chapter 1: Literature review

## 1.1 Plant-based proteins

Protein is an essential part of human diet. Consuming a balanced diet and obtaining the protein from diverse plant sources is a good strategy for the dietary-restricted population to achieve daily nutrition goals. To date, the onus of producing enough food for growing population has been residing primarily on enhanced agricultural production (Bhaskarachary et al., 2012). In the meantime, vegan and vegetarian diets have become a growing trend due to reasons such as human health, religious restrictions, and animal welfare. To fulfill this enhanced market demand, the global food industry has been continuously searching for new product formulas and emerging processing methods. Novel plant-based food products and food processing methods are being developed to fulfill both sensory and nutritional requirements of consumers. Especially in the realm of new product development, plant-based protein sources have generated lots of interest, due to their nutritional and economic advantages. Also, production of plant-based dairy alternatives has potential to significantly lower carbon emission compared to the traditional dairy industry (Westhoek et al., 2014). It also provides a delightful and nutrient-dense product for the lactose intolerant or vegan population. Food examples including soy, lentils, chickpeas, peanuts, almonds, spirulina, quinoa, chia seeds, hemp seeds, potatoes etc. are good sources of plant protein, and development of novel plant-based products incorporating them is linked to many consumer health benefits. Studies have shown that people who consume mostly plant-based food have lower body weight, cholesterol level, and blood pressure (McMacken & Shah, 2017). The risk of vegetarians having diabetes, stroke, cancer or heart disease is lower than the non-vegetarians, which may be due to the fact that a vegetable-rich diet contains a good portion of antioxidants, anti-cancer and anti-diabetic constituents (McMacken & Shah, 2017). However, many plant

protein sources are considered incomplete, since some essential amino acid is missing or is in a trace amount. Moreover, plant protein is associated with some allergic reactions. Since there is insufficient information relating to plant protein's structure, function and performance, plant protein is highly underutilized (Karaca, Low, & Nickerson, 2011).

Soy, rice and pea are the major sources of edible plant protein and have been widely used in the food industry lately (Craig, 2010). This study was mainly focused on the analysis of these three kinds of protein. Soy protein is commonly used in various kinds of food products, such as soy beverage, soy protein energy bar, soy meat alternatives, etc. It is high in essential amino acids compared to other plant proteins. Soy protein is also associated with significant decrease in serum cholesterol and, in particular, low-density lipoprotein (LDL) cholesterol concentrations (Anderson et al., 1995). The Food and Drug Administration (FDA) authorized the use of health claims that claim a role for soy protein in reducing the risk of coronary heart disease on labelling of food products that contain soy protein (FDA Talk Paper, 1999). However, soy protein is also a recognized food allergen (Hui & Sherkat, 2006). Moreover, it is deficient in the essential amino acid methionine (Friedman & Brandon, 2001).

Pea is a gluten-free and low allergenicity alternative to soy protein. Pea is cost-effective, nutrient-dense (Murat et al., 2013) and commonly grown in Canada (Stone et al., 2015). Pea is high in protein (23.1-30.9%), carbohydrate (60-65%) and low in fat (1.5-2.0%) and sodium. It also contains around 9-15% water, 2 -10% cellulose, 2-4% ash and 40-60% non-nitrogen soluble matter, including 35-40% starch, and 4-10% sugars on a dry weight basis. The composition varies among species and different growing conditions. It also contains a decent amount of vitamins and minerals (Lam et al., 2018). Pea protein is rich in lysine (3.7-6 %) but limiting in methionine (1.4-1.9%) and tryptophan (0.99-1.3%). It also contains tyrosine 2.3-3.3%, cystine 0.73-1.1%, aspartic and

glutamic acids 26-59%, histidine 2-2.6% and arginine 9.3-12.6% (% of the dry decalcified proteins). The differences in the amino acid composition are depended on the amino acid estimation method. Pea has been identified to provide multiple health benefits that include lowering plasma cholesterol and plasma triglyceride levels, thus protecting against atherosclerosis (Marchesi *et al*., 2008; Weisse *et al*., 2009). Notwithstanding this, there are several functional attributes, such as excellent gelation, water/oil-binding (Osen *et al*., 2014), foaming and emulsifying properties (Chao & Aluko, 2018), which makes pea an ideal alternative protein source for use in hypoallergenic plant-based formulated food products.

Rice protein is rich in the sulphur-containing amino acids, such as cysteine, and methionine and low in lysine. Its essential amino acid profile is considered complementary to pea protein, and often a combination of rice and pea is considered a complete protein (Woolf et al., 2011). As a result, it is commonly used along with pea proteins in various formulations. Moreover, rice protein has a distinct aroma. The aroma properties limit the application but may even be preferred by specific products and customers. Moreover, rice is also a staple cereal that is widely consumed globally, and also an important source of income for poor farmers in third world countries. Increasing dietary intake of rice protein has been associated with reduced serum cholesterol levels and overall lowering of lifestyle-related diseases (Yang et al., 2011). Rice protein is generally regarded as hypoallergenic, and several studies have highlighted the nutritional and health benefits associated with the consumption of rice protein.

### 1.2 Off-flavour from plant protein

Even though plant proteins have unlimited potential usage in food product development, the formation of undesirable green and beany flavour of pea protein highly limits their application in food product development. Flavour formation in a food product is caused by the accumulation

of aroma and taste that are affected by volatile and non-volatile compounds, respectively. The non-volatile compounds isolated from peas include 2,3-dihydro-2,5-dihydroxy-6-methyl-4 H -pyran-4-one (DDMP) saponin and saponin B, which are the most common oleanane type saponin in nature (Daveby et al., 1998). Saponins occur in a variety of plants, such as peanuts, lentils, lupins, alfalfa, and oats. Soybeans and peas are known as the most significant sources of saponins in the human diet (Oakenfull, 1981). Volatile compounds in pea mainly belong to aldehyde, ketones and alcohol groups. Moreover, the type and amount of aroma compound released are related to the protein purification level and pH level. Pea vicilin shows a high affinity for exogenous aldehyde and ketones, and pea legumin only shows affinity to the aldehyde. Pea usually contains alcohol (e.g. methanol, ethanol, and hexanol) after the harvesting, processing and storage process (Azarnia et al., 2011). Aldehyde and ketones are usually present in a smaller amount. However, it contributes to the aroma profile significantly; for instance, hexanol is usually contributed to the "hay-like" aroma in peas (Heng et al., 2004).

The unpleasant aroma of plant products is usually formed during the plant harvesting, processing and storage steps. Following reactions could all contribute to the unpleasant flavour formation (Roland et al., 2017): (1) oxidation of unsaturated fatty acid; (2) Maillard reaction between sugars and amino acid; (3) thermal degradation of phenolic acids; (4) oxidative and thermal degradation of carotenoids; (5) thermal degradation of thiamine; (6) contamination after the solvent extraction. The physicochemical parameters of the food matrix can also affect the final flavour profile; these parameters include temperature, acidity, redox potential, osmolality, salinity, and nutrient composition of the food matrix (Roland et al., 2017).

### 1.3 Lipoxygenase and their role in flavor formation

Lipoxygenase (LOX) is the major enzyme associated with aroma compound production and quality deterioration in legume (Roland et al., 2017). It catalyzes the oxidation of polyunsaturated fatty acid into fatty acid hydroperoxides. Linoleic acid and linolenic acid are the most common substrates for LOX in the plant, and they are abundant in legume and other plant cells. LOX is the primary reason for legume spoilage and quality loss. Legumes usually contain a good amount of essential fatty acid, which can be degraded by the LOX catalyzed reaction. The fatty acid deterioration involves primary autoxidation by a free radical chain reaction, photooxidation and LOX catalyzed enzymatic oxidation. Hydroperoxide is formed and further decomposed into carbonyl compounds, hydrocarbons, aldehyde, ketones and other chemicals resulted in rancidity and volatile compounds. LOX catalyzed polyunsaturated fatty acid degradation is responsible for legume senescence. These volatile compounds could be desirable and function as a natural flavouring agent, but it could also be undesirable. For example, the production of n-hexanol, n-pentanol, and n-heptanol in soybean results in a green and hay-like flavour.

Plant LOX is a one chain monomer dioxygenase enzyme (Aanangi et al., 2016), typically formed by nonheme iron, or non-sulphur iron or manganesem and a large protein structure with two different protein domains, and has a molecular weight of 90-110 kDa. The n-terminal region (approx. 25-30 kDa) consists of a beta-barrel domain, while the C-terminal is formed by α-helix with a molecular weight of 55-65 kDa. This C-terminal contains the catalytic center of LOX, which is related to iron (oxidized $Fe^{3+}$ in the active state, and reduced $Fe^{2+}$ in the inactive state, as shown in **Figure 1.1**. The catalytic iron in the active site of liquid oxygen is coordinated by five amino acid residues and a water molecule as the sixth ligand. They can specifically catalyze polyunsaturated fatty acids and esters with cis-and cis-pentadiene structure. As a result,

hydroperoxide derivatives with conjugated double bonds are formed by intramolecular oxygen addition. LOX catalyzes polyunsaturated fatty acids (PUFA) containing (1Z, 4Z)-pentadiene system into conjugated (Z, E)-diene hydroperoxy fatty acids (HPO). HPO will further degrade into metabolites and produce jasmonic acid (JA), methyl jasmonate (MJ), conjugated dienoic acids, and volatile chemicals. These compounds participate in plant defence activities, including biotic and abiotic stress regulation. (Leone, Melillo, & Bleve-Zacheo, 2001).

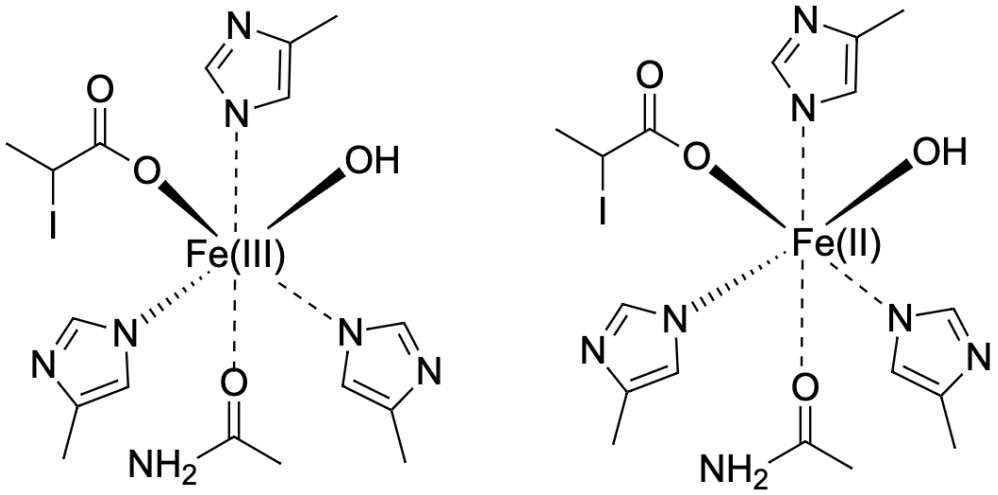



**Figure 1.1**. Active state (left) and inactive state (right) of lipoxygenase

The half-life, thermal inactivation constant, and activation energy of legume LOX has been extensively studied (Busto et al., 1999; Ludikhuyze et al., 1998; Indrawati et al., 2000). The LOX in legume can be inactivated by abiotic stress, such as thermal treatment or thermal treatment combined with high pressure or other novel processing methods. During food production and storage, optimum oxidative stability can be obtained by minimizing exposure of food products to air, light and high temperature. The stability of LOX could be also affected by the presence of additives. The turn over rate ($K_{cat}$) for LOX-1 is around 300 $sec^{-1}$, which means the reaction

happens, and the aroma is generated at an extremely fast rate. Thermal treatment is the most widely used LOX inactivation method in the industry, and the heat inactivation of pea LOX follows the 1st order kinetics (Busto et al., 1999; Ludikhuyze et al., 1998). The thermal treatment can be applied by dry heating, immersion cooking, steaming, hot grinding and extrusion cooking. Blanching at 70ºC for 2 minutes achieves >90% LOX inactivation. Emerging technologies could also be used to inactivate the LOX while maintaining the sensorial properties. These methods include (1) High-pressure processing (Indrawati et al., 2001; Akyol, Alpas, & Bayındırlı, 2006), (2) Microwave (Kermasha *et al.*, 1993; Wang *et al.*,1987), (3) Radiofrequency (Jiang et al., 2018), (4) Pulsed ultraviolet light (Janve et al., 2014), and (5) Pulsed electric field (Li et al., 2018)

**1.4 Unpleasant aroma removal method**

In order to improve the inherent flavour of plant protein ingredient, a cultivar which is initially low in off-flavour, can be selected as the food ingredient. Moreover, enzymes, like lipoxygenase (described in Section 1.3), which are associated with aroma compound production (e.g. lipoxygenase) can be inactivated before the protein extraction process. The off-flavour can be removed or modified by methods such as solvent extraction, germination, soaking or thermal treatment. For example, direct steam injection (DSI), which is one of the thermal treatment methods, has been used in the industry to remove the off-flavour from plant protein. However, high energy is been consumed during this process, and the treated product is also associated with a cooked flavour. The off-flavour can also be masked by adding flavouring into the product, such as by adding chocolate into the soymilk to make the chocolate-flavoured soymilk in order to improve the product's popularity. Volatile compounds can also be added since volatile compounds usually compete for the protein binding site.. Lactic acid fermentation (LAF) is another method

which can be used to mask the off-flavour in plant-based protein, and it is the method that has been used in this study.

### 1.5 Lactic acid fermentation

LAF is a metabolic process happening in most bacterial and animal cells, and even human muscle cells. Carbohydrate is converted to cellular energy and metabolic lactate during the lactic acid fermentation process (Makarova et al., 2006). LAF includes two kinds of processes, which are homofermentative and heterofermentative. In homofermentative bacteria like *lactococcus lactis* and *Lactobacillus plantarum*, one mole of glucose is converted to two moles of lactic acid molecules with the production of energy, carbon dioxide and ethanol. On the other hand, heterofermentative bacteria, such as *Leuconostoc mesenteroides* and *Leuconostoc dextranicum*, convert one mole of glucose into one mole of lactate, $CO_2$ and acetic acid or ethanol during the heterofermentative process. Lactic acid bacteria (LAB) are mainly Gram-positive, non-sporulating and non-motile and include both cocci and bacilli groups, which belong to Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella genera. (Mazzoli et al., 2014; Makarova et al., 2006).

LAF has been widely utilized all over the world for a long history of time in the production of wine, coffee, sourdough, yogurt, sauerkraut, pickle, kimchi, sour beer etc. Lactic acid is generally recognized as safe (GRAS) as a food ingredient by regulatory agencies, like FDA in the USA. Fermented food products are famous for their unique flavour and nutrient content. For example, fermented cereal is rich in carbohydrates, protein, fibre, minerals and vitamins. In addition, the production of metabolites of LAB influences the nutritional, texture, and organoleptic qualities of the food product. As a result, LAB is also used as food texturing agents (e.g. thickeners) and building blocks (e.g. sweeteners); Furthermore, probiotics provide many potential health

benefits to the human body. As a result, LAB has also been used as food complements. (Mazzoli et al., 2014).

Apart from the application of Lactic acid bacteria (LAB) as a food ingredient, it also has antimicrobial action, which restricts the growth of harmful microorganisms by providing stress conditions. Preservative properties of LAB are mainly achieved by the production of organic acid (including lactic acid and acetic acid) and bacteriocins (Widyastuti et al., 2014). Bacteriocins are protein or peptides which have antimicrobial activity against closely related bacterial species, and are normally produced during the primary phase of LAB growth. Nisin produced by *Lactococcus lactis* ssp. was commonly used in the food industry and is the only bacteriocin that has been approved officially worldwide (Widyastuti et al., 2014). Studies have shown that the *L.lactis* subsp. *Lactics* biovar diacetylactis successfully inhibited the growth of pathogenic *E.coli* and *Salmonella enteritidis* (Mufandaedza et al., 2006). Moreover, *Lactobacillus casei* isolated in yogurt can eliminate the growth of *Penicillium* sp. (Li et al., 2013). LAB isolated form raw milk was found to have antifungal activity against spoilage fungi including *Penicillium expansum*, *Mucor plumbeus*, *Kluyveromyces lactics* and *Pichia anomala* (Widyastuti et al., 2014).

### 1.6 Aroma generation during LAF process

LAF process produces aroma compounds that can mask the undesirable flavour in the end product like non-dairy yoghurts. The major aroma compound existing in the traditional dairy yogurt include acetaldehyde, diacetyl, propanoic acid, acetic acid, acetone, acetoin, formic acid, butanoic acid, dimethyl sulphide, benzaldehyde and 2,3-pentanedione (Widyastuti et al., 2014). Compounds belonging to different chemical groups like alcohols, aldehydes, ketones, fatty acids, esters, and sulfur compounds are associated with the aroma formed due to LAF. Functional metabolic pathways, such as the transaminase pathway, the lyase pathway, and some non-

enzymatic conversions (Smid & Kleerebezem, 2014), are responsible for the formation of these aroma compounds. Aroma formation generally includes two steps; (1) the generation of the precursor molecule and (2) the transformation of the precursor molecule into the aroma compound. Major food components such as proteins, carbohydrates and lipids serve as precursors for conversion to aroma compound, which are then transported into the cell, followed by their biochemical conversion. Not only intact, but lysed cell also contribute to aroma formation during LAF. Many cytoplasmic enzymes released by cell lysis retain their functionality outside the cell and continuously convert nutrients in the food matrix. Product formed during this process contributes to the final aroma profile (Smid & Kleerebezem, 2014). Lipid degradation, amino acid degradation, citrate metabolism, and threonine metabolism also play roles in aroma formation in the lactic acid fermentation process. The conversion of free fatty acid into secondary alcohols, organic acids and lactones contributes to the aroma profile of the product (Molimard & Spinnler, 1996). Alcohols, aldehydes, acids, esters, and sulphur compounds are formed from amino acid degradation by cytoplasmic enzymes. The citrate-driven transmission of amino acids and subsequent aroma formation pathway is shown in **Figure 1.2**. Aminotransferase converts the oxaloacetate into aspartate. In the meantime, amino acids are converted into flavour precursor α-ketoacid derivatives, diacetyl, acetoin, butanediol and acetyl aldehyde (Pudlik & Lolkema, 2012; Smid & Kleerebezem, 2014). Interestingly, citrate metabolism is also correlated with the production of aroma compounds from amino acids. Threonine metabolism is found to be correlated to the production of 2,3-pentianedione, which has a buttery, and vanilla-like aroma (Ott et al. 2000). Apart from aroma compounds, some taste-related metabolites, including amino acids (sweet, umami), oligopeptides (bitterness) and simple organic acids (sourness) (Smid & Kleerebezem, 2014), are also produced during LAF.

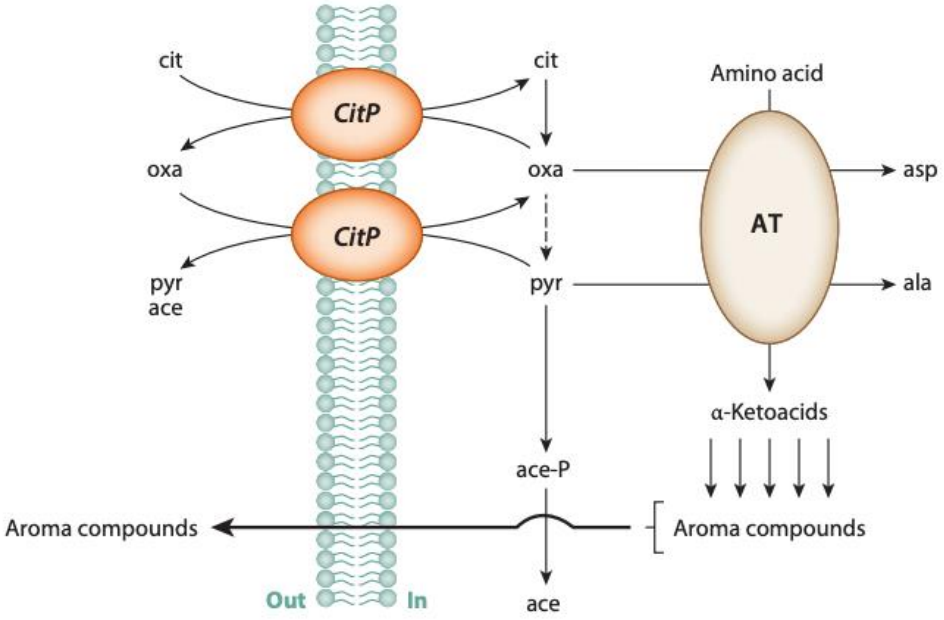



**Figure 1.2.** The aroma formation pathway driven by citrate transmission of amino acid. From "Production of aroma compounds in lactic fermentations," by Smid, E. J., & Kleerebezem, M., 2014, *Annual Review of Food Science and Technology*.

### 1.7 SPME-GC-MS

As volatile compounds are in a trace amount in the plant protein extract, so solid-phase microextraction followed by gas chromatography-mass spectrometry (SPME-GC-MS) was utilized for this analysis. Solid-phase microextraction (SPME) is widely used for trace volatile compound identification and quantification in food industries, such as the wine industry. The flowchart of analysis with SPME-GC-MS is shown in **Figure 1.3.** During the analytes extraction process, a fibre coated with an extraction phase in either liquid (polymer) or a solid (sorbent) is utilized. The concentration of the interested compound in the sample is proportional to the quantity of the compound that is extracted by the fibre. SPME could be used to extract different kinds of analytes, including both volatile and non-volatile compounds in different kinds of media, which could be in a liquid or gas phase. After the extraction, SPME fibre is transferred to the injection

port of the separating system, which is gas chromatography-mass spectrometry (GC-MS) in this study. SPME-GC-MS analysis is fast and simple, wherein the detection limit could reach parts per trillion (ppt) levels for some compounds.

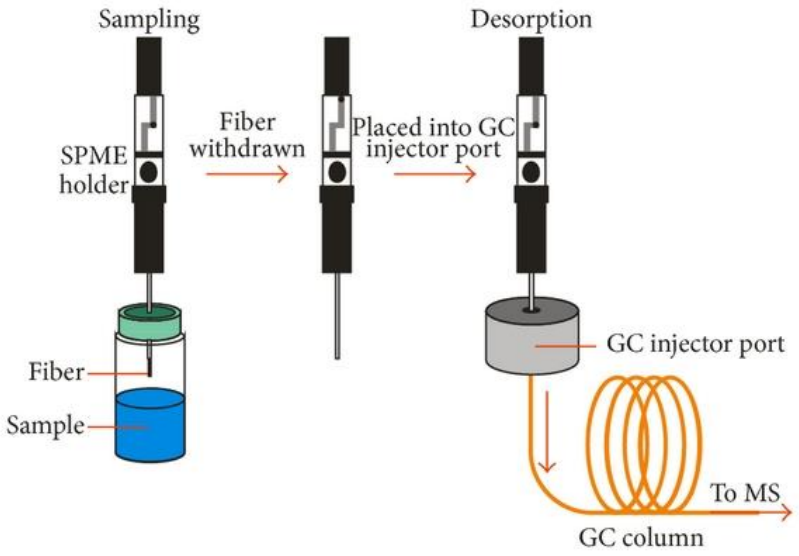



**Figure 1.3.** Analysis diagram of SPME-GC-MS. From "Current challenges in volatile organic compounds analysis as potential biomarkers of cancer," by Schmidt, K., & Podmore, I., 2015, *Journal of Biomarkers.*

However, the matrix effect exists in the sample matrix, which means that the compound in the matrix could affect the accuracy of the volatile compound detection. These compounds include carbohydrate, organic acids, amino acids, the phenolic compound, inorganic ions and proteins (Brzynski-Chang et al., 2018). In order to minimize the matrix effect, a Matrix-matched calibration curve is built by researchers. Moreover, the ratio of the concentration of the flavour compound to its odour threshold is defined as odour activity values (OAV). OAV links the chemical analysis results with sensory perception. The greater the OAV, the more aroma the compound has been contributed to the product. When the OAV is equal to or greater than one means that the compound is responsible for the final aroma profile (Zhu, Zhu, & Wang, 2015).

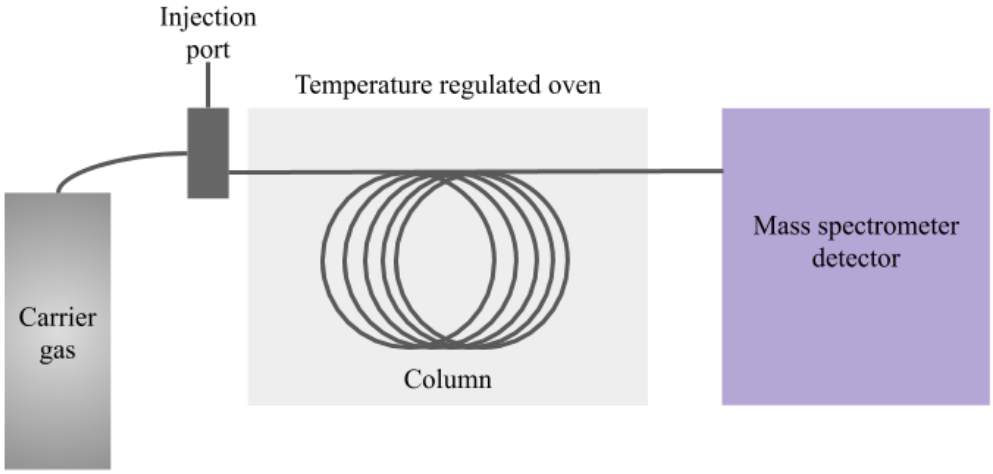



**Figure 1.4.** GC-MS schematic diagram

The schematic diagram of GC-MS system used in this study is shown in **Figure 1.4.** GC-MS is a chemical analysis technology that achieves both separation and identification. The mixture of components is separated into individual components by gas chromatography (GC), and molecules are identified by mass spectrometry (MS). Sample compounds must have sufficient volatility and thermal stability to be suitable for GC-MS analysis. Compounds must be in the gas phase or vapour phase at 250-300 ℃ or lower and do not decompose at these temperatures (Hübschmann, 2015). GC works by separating the mixture into individual substances by applying heat. The mobile phase is carrier gas, and the stationary phase is solid or heavy liquid, which is coated on the solid or support system. The sample introduced into the GC inlet is evaporated at elevated temperature, and swept into the column with a carrier gas. The sample was then separated in the column. Different affinity of components to the stationary phase leads to the separation of compounds (Hübschmann, 2015). When the instrument is running, the computer generates chromatograms according to the signal. Each peak in the chromatogram represents the signal

generated when the compound is eluted from the column to the detector, with the X-axis representing the retention time ($T_R$), and the Y-axis representing the signal strength.

After samples flow out of the chromatographic column, samples enter the MS through a the capillary column interface connecting the GC column and MS. Generally, an MS consists of four parts, which are an ion source, a high vacuum system, mass selection analyzer and an ion collector. As the sample enters MS, ion source converts the sample molecules into charged a beam of ionized particles, which are accelerated by an electric field and then passed into the mass analyzer. The unique fragmentation pattern of each compound serves as fingerprint of molecules and is used for compound determination. The ions in the gas phase are separated based on their different mass to charge ratios (m/z). Mass analyzer deflects ions down a curved tube in a magnetic field based on their energy determined by the mass, charge and velocity. The magnetic field is scanned to measure different ions. There are three types of mass analyzers, which are quadrupole, ion trap and time of flight. The detector calculates the number of ions with a specific mass, and the mass spectrum is generated. A mass spectrum is a graph of the mass to charge ratios (m/z) of the ions with different masses that travelled through the mass analyzer at the residence time T (Hübschmann, 2015). A three-dimensional graph is given by GC-MS, which includes the chromatogram of the mixture and the mass spectrum of each separated component in the chromatogram. (Hübschmann, 2015). A computer library in the computer matches the mass spectra of the samples with those in the library and identifies the unknown compound.

### 1.8 Functional properties

The physical properties of food products are highly affected by the protein component (Caballero et al., 2003). As a result, protein functionality is an important attribute to consider when implementing the protein as a food ingredient. It will affect the appearance, flavour, mouthfeel and

shelf life of the final product. The functional properties are also related to product yield and further related to the economic profit of the industry. As a result, the functional properties are essential features to consider when implementing the protein as a food ingredient. The functional properties of a protein are affected by its protein structure and hydrophobicity of their peptide and amino acid (Barac et al., 2012). Since the PPIs sample in this study are mainly used for plant-based dairy substitute production, functional properties include water/oil holding capacity, emulsifying properties and foaming properties were analyzed.

### 1.8.1 Water/Oil holding capacity

Water holding capacity and oil holding capacity represent the capability of the sample to hold water and oil. Water holding capacity is affected by the hydrogen bonding of the protein and the capability of protein to entrapment of water. Protein that is made up of majority hydrophilic group has a higher ability to absorb and retain water. If the protein contains more hydrophobic components, it will show a lower water-binding capacity but a higher oil holding capacity (Caballero et al., 2003).

### 1.8.2 Emulsifying properties

Emulsifying properties include emulsifying activity and emulsifying stability. It shows the ability of the protein to form and stabilize emulsions (Caballero et al., 2003). An emulsion is formed by dispersions of two or more immiscible liquid, which usually are water and oil in the food application, Emulsion can be stabilized by the emulsifier, which can bind the water and oil at the same time and stop the liquids from coalescing. Protein has amphipathic nature, so it can function as a natural emulsifier and stabilize the food emulsion. The rate of the protein diffusion into the interface of the water and oil fraction decides its emulsifying properties. Parameters,

including the temperature, molecular mass, matrix acidity, and the ionic strength of the protein, can all affect the diffusion rate.

### 1.8.3 Foaming properties

Protein functions as foam-forming and foam-stabilizing agents in the food matrix. Foaming in the food matrix is achieved by the formation of stable films to entrap gas. The hydrophilic part of the protein binds the water, and the hydrophobic region of the protein binds the air. As a result, a stable bridge can be formed. During the whipping process in food production, the hydrophobic region of the protein will be exposed on the interface, and partial surface denaturation happens. The partial unfolding of protein decreases the surface tension and facilitates the formation of the new interfaces and air bubbles (Caballero et al., 2003). As a result, the foaming properties is dependent on the protein diffusion rate and the ease of denaturing, which are further dependent on the protein molecular mass, surface hydrophobicity, conformation stability. The protective film formed around the air bubbles stabilizes the foam and prevent bubbles from collapsing (Caballero et al., 2003). The stability of the film and the permeability of the film to the gases together decide the foaming stability. Foams are most stable when the pH of the food matrix is close to the isoelectric point of the protein,. The small net charge near the isoelectric point promotes the association and cross-linking, promoting a more stable film. In conclusion, a protein with high solubility, small molecular weight, high surface hydrophobicity, small net charge and easy denaturability has a better capability to form and stabilize foam (Caballero et al., 2003).

## Chapter 2: Hypothesis and objectives

### 2.1 Research purpose

The study aimed to evaluate the potential of using LAF to reduce the off flavour from plant-based protein, by using solid-phase microextraction followed by gas chromatography-mass spectrometry (SPME-GC-MS) as analytical technique. This study mainly focused on measuring the evolution of aroma compounds and corresponding changes in plant protein functionality during progress of lactic acid fermentation. Descriptive analysis was performed by trained panellists to further validate a decrease in hypothesized negative attributes in PPIs by LAF treatment.

### 2.2 Research hypothesis

1. SPME-GC-MS will allow quantification of volatile compounds in plant-based protein.
2. SPME-GC-MS is useful in comparing the efficiency of different aroma removal techniques.
3. LAF is effective in reducing the unpleasant aroma from PPIs.
4. LAF could be used as a pre-treatment process in the plant-based yogurt substitute production as LAF improves the sensorial quality of PPIs.

### 2.3 Research objectives

1 Develop a methodology for comparing volatiles in plant-based protein (pea, soy and brown rice protein isolates).

2 Optimize the lactic acid fermentation process for off-flavor removal in plant-protein isolates.

3 Study the effect of LAF on protein quality.

4 Conduct a descriptive sensory analysis of LAF treated PPI samples.

## Chapter 3: Identification and quantification of the odour-active compounds in fermented and unfermented PPIs

### 3.1 Introduction

Formulating novel food products using alternative plant protein sources requires effective methods to remove or mask volatile compounds that are derived from natural, green, beany attributes that are often described by consumers as off-flavours. The origin of these off-flavours is typically associated with processing conditions that are characteristic of using spray-drying or alcohol discoloration process when fractioning or recovering protein isolates (Osen et al., 2014; Stone et al., 2015). Numerous studies have attempted to reduce the most potent odour-active volatiles in protein-sources., such as n-hexanal, a lipoxygenase-derived degradation product of linoleic acid very commonly identified in soybean (Schindler et al., 2010).

The extraction of volatiles is needed to characterize the volatile aroma profile of different plant proteins. The solid-phase microextraction (SPME) method is a simple, sensitive, robust, reliable technique. It is based on analyte diffusion that combines the advantages of both static and dynamic headspace for qualitative analysis of volatiles (Jelen, 2006). In this study, we use a simple water extraction of three distinct plant proteins to recover and identify a complex mixture of volatile compounds that can be used as signatures for pea, rice, and soy. This information will enable a better understanding of the characteristic off-flavour chemistry in these plant-based protein sources and possible strategies to be designed on how to remove them using food processing technology.

## 3.2 Materials and methods

### 3.2.1 Materials

PPIs used in this study were obtained from Daiya foods (Vancouver, Canada). Samples contained around 80% protein, 6% moisture, 5% ash, 6% carbohydrate and 8% lipids. It had cream to off-white colour and was in form of a fine powder. The sample was stored at room temperature in glass bottles and used as received. The pH of the untreated sample (control) in a 10% solution

was around 7.56. Detailed nutritional data and the amino acid profile of the PPIs sample are shown in **Table A1** and **Table A2** in appendices. Distilled and deionized water was used for all experiments in this study. The soy protein and brown rice protein used in this study were purchased from Nuts.com (Cranford, NJ, USA). The pure internal standard compound hexanal D-12 and volatile standard compounds hexanal, hexanol and 2-nonanone were purchased from Sigma-Aldrich (Canada).

### 3.2.2 Sample preparation

Sample for aroma compound extraction were prepared by dissolving 1g of powder in 7 ml of distilled deionized water inside a 20 ml glass GC vial. A 1cm-50/30 μm divinylbenzene /carboxen/polydimethylsiloxane (DVB/CAR/PDMS) Stable Flex SPME fibre was utilized for the aroma compound extraction. Prior to each sample extraction, the fibre was conditioned in injection port at 250 ℃, and the sample vial was incubated at 40℃ for 10 minutes with agitation during each extraction.

### 3.2.3 Analysis of aroma compound

After the SPME extraction, the SPME fibre was transferred into the GC injection port. The analysis in this study was performed on GC system auto-sampler (6890N Agilent) coupled to an MS detector (5963 Agilent). SPME extracts were desorbed into a DB-WAX 122-7062 capillary column (60m x 0.25mm ID, 0.25μm film thickness) using a pulsed-spitless injector, set at 250°C, for a 5 min period. Helium was used as the carrier gas at a flow rate of 1.4mL/min, and additional pressure of 30 psi was applied for 5 min. The temperature program was initially set at 40°C for 4 min, followed by an increase of 3°C/min to 150°C, and then 25°C/min to reach 230°C; after which it was kept at 230°C for 7min.

An Agilent 5973 quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV, with quadrupole temperature was set at 150 °C, and the scan ranged from 40 to 500 m/z. Data were collected using Agilent ChemStation software (standard MSD version), and volatiles recovered were searched using both NIST (v. 02) and Wiley (v. 138) libraries (Palisade Corp., Newfield, NY). Individual compounds were identified using the library search, and identities were confirmed by comparison of GC retention times with four internal standards consisting of C7–C22 n-alkanes and MS ion spectra.

The standard compounds which belong to different chemical families and the formula for the standard stock and spiking were chosen based on the preliminary SPME-GC-MS analysis results of pea samples. The compound, which shows a distinct peak in the GC chromatogram and associated with an unpleasant aroma, was chosen as the standard compound. Hexanal, 2-nonanone and hexanol were selected to represent the aldehyde, ketone and alcohol group respectively. Stock solutions of standard compounds were prepared as follows: 10000 ppm hexanal, 100 ppm 2-nonanone, 100 ppm hexanol. The standard stock was stored in a 15ml amber screw-top vials at 4ºC. The plant protein sample was spiked with 1, 2, 3, 4, 5 µl of stock standard and 5 µl of hexanal-d12 to generate the standard curve for quantification. Hexanal-d12 was used as internal standard to avoid systematic mistakes.

In order to simplify the quantification method, compounds that share the same functional group were assumed to have the same response factor. The response factor of the compound is reflected by the peak area and height in the chromatogram at a certain concentration (**Equation 3.1**), and it is highly related to the functional group of the chemical compound. As a result, all aldehyde compounds were assumed to have the same response factor as hexanal, and the concentration of all aldehyde compounds was calculated based on the standard curve of the

hexanal. All ketone compounds were assumed to share the same response factor as 2-nonanone, and 2-nonanone standard curve was used to calculate the concentration of ketone compounds. The response factor for all alcohol compounds was assumed to be the same, and the hexanol standard curve was used to calculate the concentration of all alcohol compounds. Other compounds were quantified by the response factor of aldehyde.

$$\text{Response factor} = \frac{Area\ under\ the\ peak}{Concentration} \qquad \textbf{Equation 3.1}$$

### 3.2.4 Data analysis

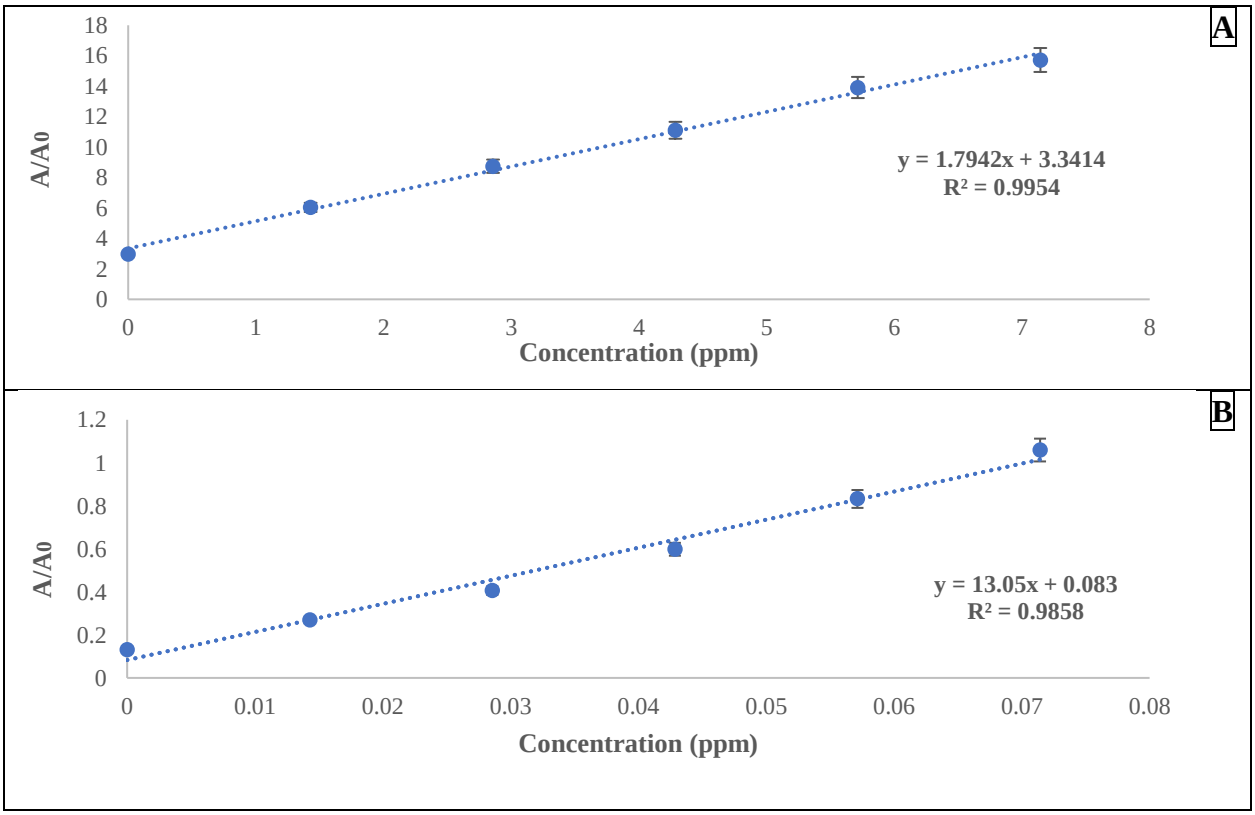
All extraction and quantification processes were performed in triplicates, and new samples were freshly prepared and used for each run. One-way analysis of variance (ANOVA) and multiple range test-Fisher's least significance of differences (LSD) at the level of p=0.05 was used to test the significant difference between samples (Excel; Microsoft Office 365 Pro Plus).

## 3.3 Results

The unpleasant aroma in the plant protein includes "beany," "green," "haylike." They are considered the limiting factor for the utilization of plant protein in the food industry (Murat, Gourrat-Pernin, Cayot, 2014). At the early stage of the study, both gas chromatography-mass spectrometry (GC-MS) and gas chromatography with flame ionization detector (GC-FID) were tried in different conditions to detect the aroma compound in the plant protein extract. However, without the solid-phase microextraction (SPME) process, GC could not successfully detect the compounds, since the volatile compounds were generated in a trace amount in the plant protein extract. As a result, solid-phase microextraction followed by gas chromatography-mass spectrometry (SPME-GC-MS) was utilized for this analysis.

### 3.3.1 Standard curve

Standard curves of hexanal, 2-nonanone, and hexanol (**Figure 3.1)** were generated and used for the aldehyde, ketone and alcohol compounds quantification. The negative x-intercept in **Figure 3.1.** A represents the original concentration of hexanal in the protein sample without spiking of additional hexanal. In order to calculate the actual concentration of the standard compound, the original concentration needs to be added to the X value during the calculation. The actual concentration of the standard compounds hexanal, nonanone and hexanol is shown in **Table 3.1**.



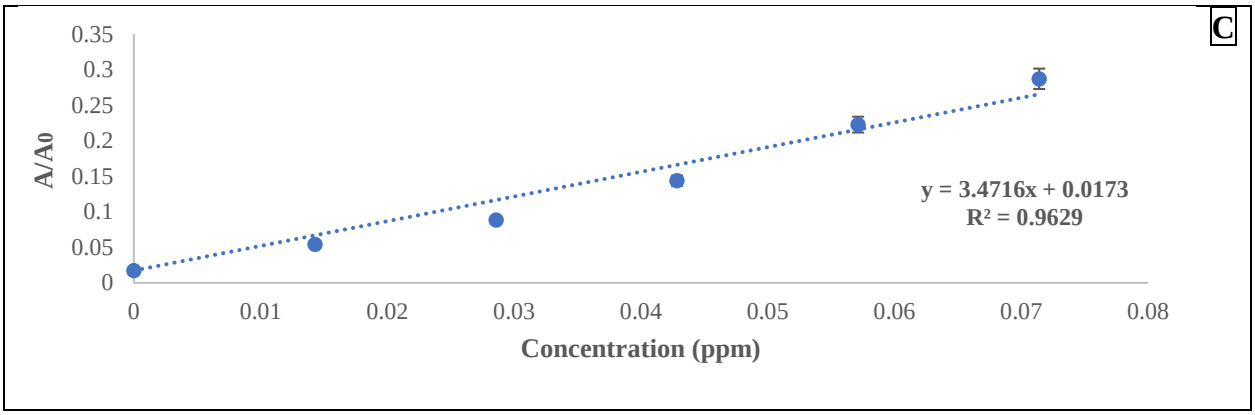



**Figure 3.1.** Standard curve of (A) hexanal, (B) 2-nonanone, and (C) hexanol. A is the chromatogram peak area of the standard compound (A: hexanal, B: 2-nonanone, and C: hexanol). A0 is the chromatogram peak area of the internal standard (hexanal-d12).

Hexanal, which has a green and grassy aroma (Burdock & Fenaroli, 2010), is a product of enzymatic or autoxidative decomposition of unsaturated fatty acids and is the most abundant aldehyde detected in pea (Azarnia et al., 2011). Similarly, 2-nonanone, which has a fresh, green, weedy and earthy smell, was present in all protein sources after the storage. Hexanol was another dominant volatile compound identified in all protein isolates and known to have an herbaceous, woody and green aroma (Murray et al., 1976; Burdock & Fenaroli, 2010). Hexanal-d12 was used as the internal standard to avoid systematic mistakes. The GC response factor is related to the functional group of the targeted compound. As a result, all compound sharing the same functional group were assumed to have the same response factor.

**Table 3.1.** The concentration of the standard compound (hexanal, 2-nonanone, and hexanol) in plant protein extracts (values expressed as mean ± standard deviation).

| | **Concentration (ppb)** | | |
|---|---|---|---|
| | **pea** | **soy** | **Brown rice** |
| **Hexanal** | 1138.00 ± 297.30 | 1621.71 ± 159.69 | 22590.24 ± 1643.70 |
| **2-Nonanone** | 6.382 ± 0.62 | n.d. | 94.02 ± 12.38 |
| **Hexanol** | n.d. | n.d. | 102.04 ± 9.30 |

Even though SPME-GC-MS has a high sensitivity, the matrix effect exists, which means that the compound in the matrix could affect the accuracy of the volatile compound detection. These compounds include carbohydrate, organic acids, amino acids, the phenolic compound, inorganic ions and proteins. These compounds might lead to the inaccuracy of the standard curve (Brzynski-Chang et al., 2018). Calibration curves for the different plant protein isolates were established and studied in this study and observed good similarity between them (**Figure B1**).

### 3.3.2 Aroma compound profile of plant protein isolates

Individual volatiles identified by corresponding retention times and linked to characteristic odours given in the literature are summarized in **Table 3.2.** Specifically, the volatile aromatic profile of the brown rice protein was complex, consisting many more different aldehydes (15), ketones (22), alcohols (11) than that recovered from soy protein which also released aldehydes (3), ketones (3) and alcohols (1), The profile of different volatiles recovered from pea protein had more similarity to soy than rice, containing aldehydes (4) and ketones (4). Aldehydes and ketones dominated the composition of volatiles recovered from the three different protein sources used in this study, which agrees with others (Schindler et al. 2012; Solina et al. 2005). Other compounds, in lower relative proportion, included alkanes and furans.

Aldehydes were found to be the most abundant volatile compounds recovered from the different protein sources and are known to be derived from either enzymatic or autoxidative decomposition of fatty acids, mainly linoleic and linolenic acids (Yi et al., 2019). Aroma intensity of aldehydes varies depending on the length of the hydrocarbon chain, as evidenced by the increase in perceived fatty odour with higher molecular weight aldehydes (Jelen & Grack, 2016). An optimal aldehyde aroma has been reported from aldehydes with 5 to 10 carbon atoms (Liu et al.,

2018). The aldehyde aroma composition profiles showed marked differences between the three proteins studied (**Table 3.2).** For example, hexanal was the major aldehyde aroma in soy (81%) and pea (90%), and brown rice (57%) proteins. Additional minor aldehydes identified in soy protein in descending concentration included benzaldehyde and pentanal, respectively. This contrasted pea protein, where benzaldehyde was the second most concentrated aldehyde, followed by pentanal and nonanal, respectively, in only trace amounts. Similarly, in addition to recovering hexanal in rice protein, there were also lower proportions of benzaldehyde, pentanal, heptanal and nonanal aldehydes, respectively**.** Hexanal, octanal and nonanal are aldehydes commonly associated with rice, along with the most recent discovery of 2-4 pentadienal (Yi et al., 2019). Hexanal, present in both soy and pea proteins, is a product of linoleic acid oxidation and has been described to contribute to the green and beany off-flavour reported in peas and soy proteins (Rackis et al., 1979). Others have reported a grassy, green and "fat-like" odour associated with pea and soy proteins, respectively (Schindler et al., 2012). Moreover, hexanal, which has a very low odour threshold (e.g. <0.005 ppm) in water (Belitz et al., 2008), is regarded as a potent recognition volatile since it can be detected at low concentrations (Reineccius & Peterson, 2013). Pentanal also has a low odour threshold of 0.012 ppm and is known for its green and milky-like odour. (Schindler et al., 2012). Benzaldehyde, another trace aldehyde (< 1% of total aldehyde concentration) detected, has a higher odour threshold of about 0.013 ppm (Yang et al., 2008). Lipid oxidation, again derived from linoleic acid substrates (Bruechert et al., 1988), and Strecker degradation of an amino acid such as phenylglycine (Vernin & Parkanyi, 1982) in vegetable protein hydrolysates (González-Pérez & Arellano, 2009), are common pathways for the production of benzaldehyde in soy and pea protein. Other aldehydes previously reported in plant proteins, but not detected in this study, included 2-nonenal, decanal and undecanal. It is feasible that the

extraction efficiency of these compounds in aqueous solvents was limited due to especially strong interactions with the protein matrix (Heng, 2005).

2-Heptanone was the major volatile ketone identified in brown rice, pea and soy protein at proportions that ranged from 37%, 24% and 83% of total ketones. In addition to 2-heptanone, brown rice also contained, in descending order, lesser proportions of ketone volatiles that included 3-octen-2-one, 2-Cyclohexen-1-one, 2-octanone, 6-undecanone, 2-nonanone, 3,5-Heptanedione, and 1-Oxaspiro (4,5) decan-2-one. This contrasted the lesser proportion of 3,5-octadien-2-one, 3-octanone and acetone, also detected. For soy protein isolates, 2-Heptanone was the major ketone present, with 2-Octanone and 3-octen-2-one also contributing to a relatively lesser amount. It is of interest that the detection of 2-heptanone and 2-nonanone, previously attributed to off-flavours described in pungent cheeses (Jelen & Grack, 2016), were recovered from pea protein; thus potentially contributing to the off-flavour of pea protein.

**Table 3.2.** Semi-quantified concentration, aromas, functional groups and retention times of various volatile compounds present in pea, soy, and brown rice protein isolates, with the concentrations of alcohols expressed in equivalent concentrations of hexanol, ketones expressed in equivalent concentrations of 2-nonanone, and aldehydes and other functional groups expressed in equivalent concentrations of hexanal.

| Chemical class | Retention time | Compound Identification | Aroma | Concentration (ppb) | | |
|---|---|---|---|---|---|---|
| | | | | Pea | Soy | Brown Rice |
| Aldehyde | 3.60 | Butanal | Pungent cocoa musty green malty bready | n.d. | n.d. | 137.19 ± 27.93 |
| | 4.50 | Pentanal | Fermented bready fruity nutty berry | 90.65 ± 13.48 | 89.12 ± 16.04 | 1837.33 ± 200.36 |
| | 5.696 | 2-Butenal, 2-ethyl | -- | n.d. | n.d. | 53.81 ± 6.53 |
| | 6.35 | Hexanal | Grassy, tallow, fatty, greeny | 1138.00 ± 297.3 | 1621.71 ± 159.69 | 22590.24 ± 1643.70 |
| | 10.93 | 2-Hexanal, 2-methyl- | -- | n.d. | n.d. | 147.85 ± 9.88 |
| | 13.22 | Octanal | Fatty, soap, lemon, green, orange, sweet | n.d. | n.d. | 981.91 ± 97.88 |
| | 14.47 | 2-Heptanal | Green type flavor | n.d. | n.d. | 134.37 ± 22.74 |
| | 14.87 | 2-Hexenal, 2-ethyl- | Fresh fruit like | n.d. | n.d. | 1717.48 ± 204.72 |
| | 15.64 | 2-Heptenal, 2-methyl- | -- | n.d. | n.d. | 1717.48 ± 204.72 |
| | 17.35 | Nonanal | Fat, citrus, greeny | 31.82 ± 5.51 | n.d. | 1129.05 ± 187.18 |
| | 18.74 | 2-Octenal | Fatty | n.d. | n.d. | 450.75 ± 47.30 |
| | 20.75 | 2-Heptenal, 2-propyl | Rosy, greeny | n.d. | n.d. | 522.22 ± 88.06 |
| | 21.24 | Benzaldehyde | Almond, burnt sugar, marchpane | 99.19 ± 4.22 | 286.96 ± 25.56 | 2798.73 ± 367.53 |
| | 24.25 | 2-Octenal, 2-butyl | -- | n.d. | n.d. | 5906.10 ± 807.06 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 28.90 | 2,4-Nonadienal | Fatty, melon, waxy, green, violet leaf, cucumber, fruit, tropical, fruit, chicken, fat | n.d. | n.d. | 126.44 ± 22.71 |
| Ketone | 3.33 | Acetone | Solvent, ethereal, apple, pear | n.d. | n.d. | 11.38 ± 1.89 |
| | 9.18 | 2-Heptanone | Soapy | 25.64 ± 8.099 | 72.63 ± 7.88 | 782.30 ± 81.94 |
| | 11.83 | 3-Octanone | Fresh, herbal, lavender, sweet, mushroom | n.d. | n.d. | 25.31 ± 1.51 |
| | 13.01 | 2-Octanone | Soapy, fruity | n.d. | 2.86 ± 2.51 | 109.19 ±11.15 |
| | 14.61 | 3-Ethylcyclopentanone | Meat-like | n.d. | n.d. | 33.41 ± 3.03 |
| | 15.05 | Cyclohexanone, 4-ethyl- | -- | n.d. | n.d. | 20.96 ± 3.10 |
| | 17.24 | 2-Nonanone | Hot milk, soapy, greeny | 6.38 ± 0.62 | n.d. | 94.02 ±12.38 |
| | 17.82 | 3-Octen-2-one | Mushroom, mouldy, burnt | n.d. | 12.03 ± 0.17 | 389.34 ± 46.50 |
| | 18.90 | 5-Decanone | Fermented | n.d. | n.d. | 34.98 ± 4.65 |
| | 19.83 | Ethanone[x] | -- | 10.04 ± 3.653 | n.d. | 19.44 ± 33.67 |
| | 22.90 | 6-Undecanone | -- | n.d. | n.d. | 94.74 ± 9.57 |
| | 23.87 | 3, 5-Octadien-2-one[x] | Fruity, fatty, mushroom like | 48.71 ± 5.575 | n.d. | 30.40 ± 2.82 |
| | 26.65 | 2-Cyclohexen-1-one, 2-hydroxy-6-methyl-3-(1-methylethyl)- | -- | n.d. | n.d. | 149.72 ± 21.73 |
| | 26.276 | 6,6,7-Trimethyl-octane-2,5-dione | -- | n.d. | n.d. | 45.68 ± 8.00 |
| | 26.53 | 3,5-Heptanedione, 2,2,6,6-tetramethyl- | -- | n.d. | n.d. | 5.03 ± 1.32 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 26.80 | 6-Dodecanone | Fruity citrus floral orange | n.d. | n.d. | 17.5 ± 1.89 |
| | 30.16 | 3,5-Heptanedione, 2,2,6,6-tetramethyl- | -- | n.d. | n.d. | 51.51 ± 5.81 |
| | 31.58 | 1-Oxaspiro(4,5)decan-2-one | -- | n.d. | n.d. | 39.16 ± 5.61 |
| | 32.18 | 5H-Inden-5-one, 1,2,3,6,7,7a-hexahydro-7a-methyl- | -- | n.d. | n.d. | 7.29 ± 0.72 |
| | 32.83 | 2-Isopropyl-5,5-dimethylcyclohex-2-enone | -- | n.d. | n.d. | 12.77 ± 1.58 |
| | 33.35 | 2-(2-Methyl-propenyl)-cyclohexanone | -- | n.d. | n.d. | 5.82 ± 1.19 |
| | 33.55 | 2(3H)-Furanone, dihydro-5-pentyl- | -- | n.d. | n.d. | 18.49 ± 2.74 |
| Alcohol | 11.45 | 1-pentanol | Fruity | n.d. | n.d. | 1171.26 ± 31.66 |
| | 15.66 | 1-Hexanol | Resin, flowery, greeny | n.d. | n.d. | 102.04 ± 9.30 |
| | 19.43 | 1-Octen-3-ol | Mushroomy, earthy, burnt | n.d. | 40.21 ± 9.61 | 1850.47 ± 221.00 |
| | 19.71 | 1-Heptanol | Greeny | n.d. | n.d. | 421.46 ± 44.14 |
| | 19.89 | Bicyclo[2,2,2]octan-1-ol, 2-methyl | -- | n.d. | n.d. | 125.05 ± 111.33 |
| | 21.61 | 2,4-Pentadien-1-ol, 3-pentyl- | -- | n.d. | n.d. | 121.15 ± 11.04 |
| | 21.89 | 3-Nonen-2-ol | Fruity berry fatty oily ketonic weedy spicy licorice | n.d. | n.d. | 263.69 ± 26.34 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 22.58 | Cyclohexanol, 2,4-dimethyl- | -- | n.d. | n.d. | 118.36 ± 10.74 |
| | 22.65 | 4-Ethylcyclohexanol | -- | n.d. | n.d. | 89.24 ± 56.36 |
| | 23.71 | 1-Octanol | Waxy green orange aldehydic rose mushroom | n.d. | n.d. | 397.56 ± 56.36 |
| | 33.79 | 4,4,6-Trimethyl-cyclohex-2-en-1-ol | -- | n.d. | n.d. | 192.21 ± 29.43 |
| Other functional groups | 2.91 | Carbon dioxide | No odour at low concentration, sharp acidic smell at high concentration | 15.91 ± 8.01 | 30.81 ± 10.42 | 42.48 ± 9.29 |
| | 2.94 | Pentane | Gasoline-like | n.d. | 60.36 ± 23.56 | 79.43 ± 4.97 |
| | 3.12 | Heptane | Petroleum-like | n.d. | n.d. | 167.98 ± 36.44 |
| | 2.30 | Hexane | Gasoline-like | n.d. | 25.59 ± 13.80 | n.d. |
| | 3.40 | Octane | Green, minty, herbal, rosemary, cooling | 25.48 ± 23.98 | 29.67 ± 15.60 | 652.63 ± 140.77 |
| | 4.12 | Furan, 2-ethyl | Chemical, beany, bready, malty | n.d. | 108.76 ± 34.25 | n.d. |
| | 4.50 | Pentanal | Fermented, bready, fruity, nutty, berry. | n.d. | 89.12 ± 16.04 | n.d. |
| | 5.30 | Toluene | Sweet | n.d. | n.d. | 141.19 ± 17.08 |
| | 5.41 | Decane | Gasoline-like | 15.88 ± 25.91 | n.d. | 89.78 ± 7.51 |
| | 5.79 | Disulfide, dimethyl | Garlic-like | n.d. | n.d. | 302.73 ± 39.60 |

| | | | | | |
|---|---|---|---|---|---|
| 7.12 | 2-n-Butyl furan | Mild, fruity, wine, sweet, spicy | n.d. | n.d. | 386.40 ± 30.64 |
| 7.61 | Undecane | Gasoline-like to Odorless | 35.32 ± 62.71 | n.d. | n.d. |
| 8.10 | 5-Undecene | -- | n.d. | n.d. | 1410.36 ± 296.77 |
| 10.52 | Furan, 2-pentyl | Fruity, green, earthy, beany, vegetable, metallic | 638.17 ± 405.83 | 2492.34 ± 199.48 | n.d. |
| 10.61 | Cyclohexene, 3-butyl- | -- | n.d. | n.d. | 120.13 ± 104.12 |
| 11.42 | Dodecane | -- | 77.93 ± 59.84 | n.d. | n.d. |
| 12.75 | Hexanenitrile | -- | n.d. | n.d. | 113.44 ± 10.41 |
| 15.46 | Dimethyl trisulfide | Sulfurous, onion, cooked, onion, savory, meaty | n.d. | n.d. | 273.51 ± 43.22 |
| 17.06 | Hexanethioic acid, S-methyl ester | Fruity, ethereal, green, tropical, cabbage, cheesy, rancid, floral, green | n.d. | n.d. | 169.03 ± 21.49 |
| 18.44 | 5-Tridecene | -- | n.d. | n.d. | 137.54 ± 14.33 |
| 28.80 | Pentanoic acid | Acidic, sweaty, rancid | n.d. | n.d. | 225.04 ± 47.34 |
| 28.90 | 2,4-Nonadienal | Fatty, melon, waxy, green, violet, leaf, cucumber, fruit, tropical, fruit, chicken, fat | n.d. | n.d. | 126.44 ± 22.71 |
| 32.04 | α-D-Glucopyranoside, O-α-D-glucopyranosyl- | -- | n.d. | n.d. | 37.84 ± 33.30 |

| | | | | | |
|---|---|---|---|---|---|
| | (1.fwdarw.3)-β-D-fructofuranosyl | | | | |
| 32.47 | E-2-Hexenyl E-2-octenoate | Fruity, pear, skin, green, waxy, tropical, plum, skin, fatty | n.d. | n.d. | 19.83 ± 18.11 |
| 32.75 | Oxime-, methoxy-phenyl-_ | -- | n.d. | 16.67 ± 4.38 | n.d. |
| 32.89 | Heptanoic acid | Rancid, sour, cheesy, sweaty | n.d. | n.d. | 123.71 ± 108.23 |
| 33.08 | 1,2-Diazaspiro(2.5)octane | -- | n.d. | n.d. | 51.54 ± 7.64 |

Concentrations expressed in equivalent ppb concentrations of Hexanol for alcohols, of 2-nonanone for ketones, and of Hexanal for aldehydes and other functional groups
The results shown are the average of triplicate assessments and are expressed as mean ± standard deviation.
Components are listed on the order of their chemical family.
All compounds were identified by comparison with mass spectra and retention index database.
n.d., not detected, below limit of quantification.

1-Octen-3-ol, another product of lipid oxidation, derived from lipoxygenase activity of linoleic acid precursors, was recovered in soy and brown rice protein. The only alcohol compound existed in soy protein sample was 1-octen-3-ol, which contributed to 37% of alcohols in brown rice. Others have characterized 1-octen-3-ol to have a low odour threshold (e.g. 0.001 ppm) with a distinct mushroom odour (Schindler et al., 2012). The alcohol volatiles derived from rice protein were more complex containing 1-Octen-3-ol as the principal component, with lesser proportions of 1-pentanol, 1-heptanol and 1-octanol, respectively.

Furans, formerly attributed to the grassy-beany and green flavour in pea protein (Reineccius, 2018), was an additional class of volatile aromas recovered from the three different plant proteins studied. Pea and brown rice protein contained only one kind of furan, which was 2-pentyl furan and 2-n-Butyl furan, respectively. In soy, both 2-ethyl furan (4%) and 2-pentyl furan (96%) exist. Some alkanes and alkenes were also identified in all protein sources studied; however, these volatiles do not have a major role in generating off-flavours. Indeed, pure alkanes are considered tasteless and nearly odourless.

The total concentration of aldehyde, ketone and alcohol compounds is shown in **Table 3.3**. Pea contained the lowest level of aldehyde compounds, and no alcohol compounds were detected in the pea sample. The lowest level of ketone compounds was detected in soy samples. The highest amount of aldehyde, ketone and alcohol compounds was detected in brown rice sample

**Table 3.3.** The concentration of total aldehyde, ketone, and alcohol compounds in plant protein extracts (values expressed as mean ± standard deviation)

| | **Concentration (ppb)** | | |
|---|---|---|---|
| | **pea** | **soy** | **Brown rice** |
| **Aldehyde** | 1359.70 ± 102.03 | 1997.78 ± 200.42 | 38650.84 ± 3674.23 |
| **Ketone** | 107.97 ± 4.64 | 87.52 ± 6.28 | 2120.82 ± 271.40 |

| | | | |
|---|---|---|---|
| **Alcohol** | n.d. | 40.21 ± 9.61 | 4996.69 ± 505.20 |

### 3.4 Conclusion

In this study, volatile compounds of plant-based protein were first recovered by water extraction. Solid-phase micro-extraction coupled with gas chromatography-mass spectrometry (SPME-GC-MS) was then utilized to successfully identify and quantify the volatile compounds in pea, soy and brown rice protein sample. The volatiles profile of varying protein samples was studied and compared. Pea and soy protein sample had a comparable aroma profile. However, the content of unpleasant volatile in brown rice extract was significantly higher. The SPME-GC-MS method will be useful in detecting the aroma compound profile of different protein samples. It could also be used to follow the evolation of volatiles on application of various aroma removal and other food processing techniques.

# Chapter 4: Optimization of the lactic acid fermentation process for production of plant protein isolates

## 4.1 Introduction

Pea is a nutrient-dense and cost-effective protein source. It also delivers good functional properties to be used as a food ingredient. The application of pea as a food ingredient is limited by the persistent greeny aroma. The purpose of this study was to evaluate the potential of LAF in eliminating the unpleasant aroma form PPIs. The aroma profiles of PPIs that were fermented with three groups of lactic acid-producing bacterias were studied and compared. Moreover, the LAF treatment was optimized by comparing the SPME-GC-MS results achieved by different bacteria strains and fermentation time.

## 4.2 Materials and methods

### 4.2.1 Materials

Tuzen® *Lactobacillus plantarum* 299v was purchased from local drug mart (Vancouver, Canada). *Lactobacillus casei* strain was obtained from the University of British Columbia (UBC) food safety & health engineering laboratory (Vancouver, Canada). Life brand$^{TM}$ probiotic capsules were purchased from local Drug Mart (Vancouver, Canada), and each of the probiotic capsules contained 6 billion active cells of the following strains: 50% (3.0 billion CFU) *Lactobacillus rhamnosus*, 30% (1.8 billion CFU) *Lactobacillus casei*, 10% (0.6 billion CFU) *Lactobacillus acidophilus*, and 10% (0.6 billion CFU) *Bifidobacterium longum*. The MRS agar and MRS broth used for microbiology studies were purchased from Sigma-Aldrich (Canada). Distilled deionized water from the UBC lab was used to prepare the protein suspension.

### 4.2.2 Lactic acid fermentation

The effectiveness of different bacteria strains on aroma compound removal was compared. Three groups of 10% PPIs solution were inoculated with three groups of different bacteria at 7 Log CFU/ml. Group A sample was inoculated with *Lactobacillus plantarum*, group B pea protein sample was inoculated with *Lactobacillus casei*, and group C sample was inoculated with four mix strains of probiotics (Life brand$^{TM}$). The fermentation was conducted on a 37 °C anaerobic environment, and the fermentation was terminated by freezing the sample at -20°C. The frozen sample was freeze-dried by the bulk try dryer (Labconco) into powder form and stored at -4°C for further aroma analysis.

The PPIs sample, which has been fermented for 5, 10 and 20 hours were analyzed and compared. The same aroma compound detection and quantification methods in the previous chapter were used to analyze the fermented PPIs sample. After the optimum strain for off-flavour removal was selected, fermentation was conducted again by using the optimum strain. The detailed LAF treatment by the optimum strain was analyzed. The measurement of aroma profile was performed after 5, 10, 15, 20 and 25 hours of fermentation.

After the fermentation treatment, the pH of PPIs solution was measured by pH meter (Fisher Scientific). MRS broth was used for dilution of samples. Diluted sample was plated on MRS agar plate and inoculated at 37ºC under anaerobic condition to generate the number of CFU/ml in the fermented sample.

### 4.2.3 Analysis of aroma compound

The same aroma analysis method which was used in section 3 was used in section 4. Seven ml of distilled deionized water was added into 1 g of freeze-dried sample powder right before the aroma analysis. The aroma profiles of PPIs samples that has been fermented for a different amount of time were compared.

### 4.2.4 Data analysis

All fermentation and aroma quantification were performed in triplicate. The significant difference between samples were tested by one-way analysis of variance (ANOVA) and multiple range test-Fisher's least significance of differences (LSD) at the level of p=0.05 (Excel; Microsoft Office 365 Pro Plus).

## 4.3 Results and discussion

### 4.3.1 Lactic acid fermentation with different bacteria strains

*Lactobacillus casei* (Group B) and mix strains of probiotic (Group C) fermented samples had the same creamy colour as the untreated pea protein sample. However, the colour of *Lactobacillus plantarum* fermented sample (Group A) showed a darker and yellower colour than the control (unfermented). The metabolism of different lactic acid-producing bacteria leads to differences in the final colour appearance.

The aroma compounds identities and concentration of PPIs samples that have been fermented with different bacteria strains in different fermentation times are shown in **Table 4.1** (*L. plantarum*), **Table 4.2** (*L. casei*), and **Table 4.3** (mix strains of probiotics). Volatile compound profiles of the control (time 0) group sample in **Table 4.1**, **Table 4.2**, and **Table 4.3** were not the same. Eight types of aldehyde compounds were detected in the control in **Table 4.1**. However, six types of aldehyde compounds were detected in the control in **Table 4.2**, and five types of aldehyde compounds were detected in the control in **Table 4.3**. Butanal (62 ppb) and 2-heptanal (15 ppb) was only existed in control of **Table 4.1**, but the control of **Table 4.2** and **Table 4.3** do not contain butanal and 2-heptanal. Moreover, the control in **Table 4.1** contains six kinds of ketone (2-heptenone, 2-octanone, 2-nonanone, 2-decanone, 3,5-octadien-2-one, and ethenone) and four types of alcohol compounds (1-pentanol, 4,4-dimethyl-cyclohex-2-en-1-ol, 1-octen-3-ol, and 1-

octanol). The control in **Table 4.2** had four types of ketone compounds (2-heptanone, 2-nonanone, 3,5-octadien-2-one, and ethenone) and only one kinds of alcohol compounds (1-octen-3-ol). Three kinds of ketone compounds (2-heptaonone, 3,5-octandien-2-one, and ethenone) and three types of alcohol compounds (1-pentanol, 3,5-octadien-2-ol, and 1-octadien-2-ol) were found in the control in **Table 4.3**. The control in each table were generated from different batches of sample, and the sensitivity of SPME-GC-MS was high. As a result, trace amount of difference in aroma profile could be detected. The volatile compounds profile of PPIs was affected by the cultivar, crop year, and processing conditions (Azarnia et al., 2011). Nevertheless, the storage time and condition could all affect the volatile compound profile. According to Schindler (2012), a slight increase of n-hexanal concentration was observed in pea protein during the storage. The formation of aldehyde during storage is caused by the enzymatic or autoxidative decomposition of unsaturated fatty acids (Azarnia et al., 2011). As a result, a different control group needs to be run for each batch of samples. Since difference existed in the aroma profile of controls, the relative change of the aroma compounds in percentage (**Figure 4.1**) after the treatments was analyzed.

Based on results shown in **Table 4.1**, **Table 4.2**, and **Table 4.3**, the fermentation process altered the aroma profile of protein significantly, which was consistent with the results generated by Schindler et al. (2012). LAF altered the volatile compounds profile of PPIs by either reduce the volatile compound or masking the off-flavours (Schindler et al., 2012). Some of the aroma compound, especially aldehyde compounds lost the binding with the PPIs and evaporated during the fermentation process. As shown in **Table 4.1**, **Table 4.2** and **Table 4.3**, aldehyde compounds including, pentanal, benzaldehyde, octanal, and nonanal were no longer detectable after 20 hours of fermentation. Alcohol compounds were produced during the fermentation process. After 20 hours of LAF, alcohol compounds including 1-hexanol and 1-octen-3-ol were produced in all three

groups of samples. The alcohol compounds that has been produced could compete with aldehyde compounds for protein binding sites, and aldehyde compound which have a lower affinity to the protein would lose the binding. The binding between protein and aroma compound was affected by the ionic strength, the specific flavour compound and pH of the matrix (Wang & Arntfield, 2015). As a result, the environment of the solvent can affect the binding between protein and flavour compound significantly. During the LAF, lactic acid was produced, and the pH of the PPIs solution decreased. Based on Wang and Arntfield (2015), the flavour binding affinity between aroma compounds and protein was found to be lower at acidic environment. As a result, some of the volatile compounds were no longer exist after the LAF treatment.

**Table 4.1** Aldehyde, ketone and alcohol compounds concentration (**ppb**) detected in *Lactobacillus plantarum* fermented PPIs by using SPME-GC-MS.

| Chemical class | Retention time (min) | Compound identification | Fermentation time (hours) | | | |
|---|---|---|---|---|---|---|
| | | | **0** | **5** | **10** | **20** |
| **Aldehyde** | 3.60 | Butanal | 61.78 ± 0.28 | n.d. | n.d. | n.d. |
| | 4.50 | Pentanal | 203.12 ± 0.93 | 65.47 ± 0.30 | 57.63 ± 0.27 | n.d. |
| | 6.07 | Benzaldehyde, 2,4-bis(trimethylsiloxy)- | 16.81 ± 0.08 | n.d. | 34.34 ± 0.16 | n.d. |
| | 6.33 | Hexanal | 3371.37 ± 15.51 | 1063.38 ± 4.89 | 751.85 ±3.46 | 423.73 ± 1.95 |
| | 13.18 | Octanal | 70.94 ± 0.33 | 38.05 ± 0.18 | 21.49 ± 0.10 | n.d. |
| | 14.47 | 2-Heptenal, (Z)- | 14.56 ± 0.07 | n.d. | n.d. | n.d. |
| | 17.41 | Nonanal | 70.15 ± 0.32 | 54.82 ± 0.25 | 71.45 ± 0.33 | n.d. |
| | 21.35 | Benzaldehyde | 114.13 ± 0.53 | 33.38 ± 0.15 | 37.37 ± 0.17 | n.d. |
| **Ketone** | 8.28 | 3-Heptanone | n.d. | 5.27 ± 0.07 | n.d. | n.d. |
| | 8.31 | 3-Hexanone, 5-methyl- | n.d. | n.d. | 6.24 ± 0.09 | n.d. |
| | 9.23 | 2-Heptanone | 76.16 ± 1.08 | 33.17 ± 0.47 | 29.17 ± 0.41 | 44.94 ± 0.64 |
| | 11.73 | 2-Butanone, 3-hydroxy- | n.d. | n.d. | 4.19 ± 0.06 | n.d. |
| | 13.05 | 2-Octanone | 0.40 ± 0.01 | 2.77 ± 0.04 | 2.86 ± 0.04 | n.d. |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 17.25 | 2-Nonanone | 10.70 ± 0.15 | 9.43 ± 0.13 | 7.67 ± 0.11 | 11.19 ± 0.16 |
| | 21.44 | 2-Decanone | 11.35 ± 0.16 | 8.63 ± 0.12 | 8.07 ± 0.11 | 12.68 ± 0.18 |
| | 23.91 | 3,5-Octadien-2-one and isomers | 27.48 ± 0.39 | 11.50 ± 0.16 | 14.02 ± 0.20 | 7.76 ± 0.11 |
| | 26.28 | Ethanone, 1-(3-butyloxiranyl)- | 10.73 ± 0.15 | 8.45 ± 0.12 | 8.66 ± 0.12 | 11.10 ± 0.16 |
| **Alcohol** | 3.82 | 1-Heptanol, 2-propyl- | n.d. | 8.11 ± 0.30 | n.d. | n.d. |
| | 11.34 | 1-Pentanol | 15.89 ± 0.59 | 9.00 ± 0.33 | 25.17 ± 0.93 | n.d. |
| | 14.80 | 4,4-Dimethyl-cyclohex-2-en-1-ol | 31.01 ± 1.15 | n.d. | n.d. | n.d. |
| | 15.41 | 1-Hexanol | n.d. | 151.41 ± 5.62 | 240.12 ± 8.91 | 346.57 ± 12.86 |
| | 19.24 | 1-Octen-3-ol | 71.29 ± 2.64 | 19.51 ± 0.72 | 31.68 ± 1.18 | 30.00 ± 1.11 |
| | 23.56 | 1-Octanol | 8.88 ± 0.33 | n.d. | n.d. | n.d. |

The results shown are the average of triplicate assessments and are expressed as mean ± standard deviation.
Components are listed on the order of their chemical family.
All compounds were identified by comparison with mass spectra and retention index database.
n.d., not detected, below limit of quantification.

**Table 4.2.** Aldehyde, ketone and alcohol compounds detected in *Lactobacillus casei* fermented pea protein isolates powder by using SPME-GC-MS.

| Chemical class | Retention time (min) | Compound Identification | Fermentation time (hours) | | | |
|---|---|---|---|---|---|---|
| | | | **0** | **5** | **10** | **20** |
| **Aldehyde** | 4.54 | Pentanal | 54.94 ± 0.25 | 56.84 ± 0.26 | 5.14 ± 0.02 | n.d. |
| | 6.08 | Benzaldehyde, 2,5-bis[(trimethylsilyl)oxy]- | 19.64 ± 0.09 | n.d. | n.d. | n.d. |
| | 6.33 | Hexanal | 1003.08 ± 4.61 | 1176.29 ± 5.41 | 995.34 ± 4.58 | 267.58 ± 1.23 |
| | 13.27 | Octanal | 16.29 ± 0.07 | 22.33 ± 0.10 | n.d. | n.d. |
| | 17.48 | Nonanal | 37.18 ± 0.17 | 40.87 ± 0.19 | n.d. | n.d. |
| | 21.49 | Benzaldehyde | 76.26 ± 0.35 | 74.17 ± 0.34 | 34.46 ± 0.16 | n.d. |
| **Ketone** | 4.15 | (3R)-3-Phenyl-2,3-dihydro-1H-isoindol-1-one | n.d. | n.d. | n.d. | 12.52 ± 0.17 |
| | 9.31 | 2-Heptanone | 13.96 ± 0.20 | 16.20 ± 0.23 | 17.48 ± 0.25 | 6.60 ± 0.09 |
| | 17.33 | 2-Nonanone | 2.60 ± 0.04 | 3.47 ± 0.05 | n.d. | 2.70 ± 0.04 |
| | 21.53 | 2-Decanone | n.d. | n.d. | n.d. | 3.70 ± 0.05 |
| | 24.02 | 3,5-Octadien-2-one and isomers | 28.90 ± 0.23 | 19.45 ± 0.21 | 15.38 ± 0.22 | 8.68 ± 0.12 |
| | 24.41 | Ethanone, 1-(3-butyloxiranyl)- | 5.46 ± 0.08 | n.d. | n.d. | n.d. |
| | 26.39 | Acetophenone | n.d. | 19.66 ± 0.28 | 16.23 ± 0.23 | 18.65 ± 0.27 |
| **Alcohol** | 7.92 | 1-Butanol | n.d. | n.d. | n.d. | 4.58 ± 0.17 |
| | 11.48 | 1-Pentanol (CAS) | n.d. | n.d. | n.d. | 5.13 ± 0.19 |
| | 15.54 | 1-Hexanol | n.d | n.d. | n.d. | 68.42 ± 2.54 |

| | | | | | |
|---|---|---|---|---|---|
| 19.39 | 1-Octen-3-ol | 11.47 ± 0.43 | 17.80 ± 0.66 | 20.22 ± 0.75 | 14.99 ± 0.56 |

The results shown are the average of triplicate assessments and are expressed as mean ± standard deviation.
Components are listed on the order of their chemical family.
All compounds were identified by comparison with mass spectra and retention index database.
n.d., not detected, below limit of quantification.

**Table 4.3.** Aldehyde, ketone and alcohol compounds concentration (ppb) detected in mix strains of probiotic fermented PPIs by using SPME-GC-MS.

| Chemical class | Retention time (min) | Compound Identification | Fermentation time (h) | | | |
|---|---|---|---|---|---|---|
| | | | **0 hr** | **5 hr** | **10hr** | **20hr** |
| **Aldehyde** | 4.53 | Pentanal | 58.69 ± 0.27 | 39.48 ± 0.18 | n.d. | n.d. |
| | 6.32 | Hexanal | 1242.00 ± 5.71 | 483.40 ± 2.22 | 233.50 ± 1.07 | 256.20 ± 1.18 |
| | 13.23 | Octanal | 23.16 ± 0.11 | 13.65 ± 0.06 | n.d. | n.d. |
| | 17.47 | Nonanal | 41.32 ± 0.19 | 32.57 ± 0.15 | n.d. | n.d. |
| | 21.46 | Benzaldehyde | 106.50 ± 0.49 | 66.98 ± 0.31 | 41.58 ± 0.19 | n.d. |
| **Ketone** | 4.15 | (3R)-3-Phenyl-2,3-dihydro-1H-isoindol-1-one | 1.26 ± 0.18 | 7.00 ± 0.10 | 8.01 ± 0.11 | n.d. |
| | 5.35 | 2-Butanone, 3,3-dimethyl-1-(methylsulfonyl)-, O-[(methylamino)carbonyl] oxime | n.d. | 16.19 ± 0.23 | 23.06 ± 0.33 | 45.05 ± 0.64 |
| | 5.66 | 2-Propanone, 1-phenyl- | n.d. | n.d. | n.d. | n.d. |
| | 9.29 | 2-Heptanone | 13.39 ± 0.19 | 15.17 ± 0. 22 | 7.49 ± 0.11 | 13.58 ± 0.19 |
| | 9.30 | 2-Butanone, 3-hydroxy- | n.d. | n.d. | n.d. | n.d. |
| | 17.31 | 2-Nonanone | n.d. | 2.96 ± 0.04 | 1.98 ± 0.03 | 4.33 ± 0.06 |
| | 24.00 | 3,5-Octadien-2-one[x] | 30.17 ± 0.23 | 21.98 ± 0.217 | 13.31 ± 0.15 | 13.86 ± 0.20 |
| | 26.39 | Ethanone, 1-(3-butyloxiranyl)- | 1.94 ± 0.03 | n.d. | n.d. | 4.79 ± 0.07 |
| **Alcohol** | 11.50 | 1-Pentanol | 9.16 ± 0.34 | 20.46 ± 0.76 | 14.59 ± 0.54 | 29.59 ± 1.10 |

| | | | | | |
|---|---|---|---|---|---|
| 15.72 | 1-Hexanol | n.d. | 231.28 ± 8.58 | 189.46 ±7.03 | 353.30 ± 13.11 |
| 17.85 | 3,5-Octadien-2-ol | 12.24 ± 0.45 | n.d. | n.d. | n.d. |
| 19.37 | 1-Octen-3-ol | 13.96 ± 0.52 | 16.74 ± 0.62 | 12.42 ± 0.46 | 18.65 ± 0.69 |
| 19.64 | 1-Heptanol | n.d. | 7.26 ± 0.27 | 6.19 ± 0.23 | n.d. |
| 23.67 | 1-Octanol | n.d. | 4.86 ± 0.18 | 5.26 ± 0.20 | n.d. |
| 26.38 | 4-Hexen-3-ol, 4-Hexen-3-ol, 2,5-dimethyl- | n.d. | n.d. | 13.71 ± 0.51 | n.d. |
| 27.52 | 1-Nonanol | n.d. | n.d. | n.d. | n.d. |

The results shown are the average of triplicate assessments and are expressed as mean ± standard deviation.
Components are listed on the order of their chemical family.
All compounds were identified by comparison with mass spectra and retention index database.
n.d., not detected, below limit of quantification.

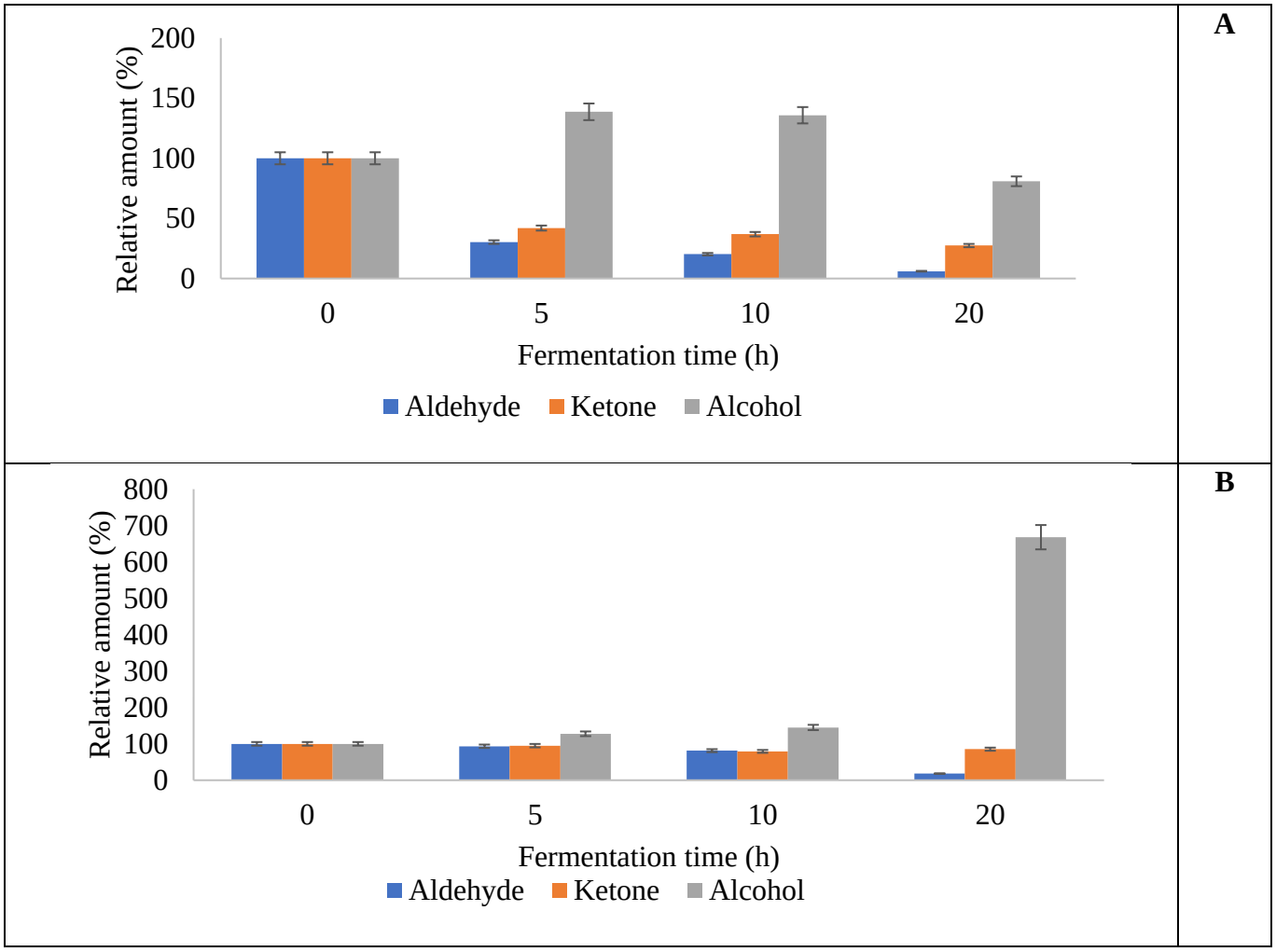
**Figure 4.1** shows the total relative amount of volatile compound (aldehyde, ketone, and alcohol) concentration of PPIs samples, which have been fermented with (A) *Lactobacillus plantarum,* (B) *Lactobacillus casei,* and (C) mix strains of probiotics in 0, 5, 10, and 20 hours. The aroma compound concentration in control (untreated) sample was 100%.

According to the result, the relative amount of total aldehyde decreased with the fermentation treatment time for all groups of samples. As a result, the *L. plantarum, L. casei* and mix strains of probiotics fermentation all successfully removed aldehyde compound in the pea protein powder and the effectiveness of aldehyde removal was proportional to the fermentation time. *L. plantarum* achieved the highest amount of total aldehyde reduction in all time points of measurement (5, 10 and 20 hours).

For all three groups of samples, ketone concentration was first decreased and then increased along with the fermentation time. After 20 hours of fermentation, the ketone concentration has increased for all samples. This is because new ketone compounds have been produced by LAF. The best ketone removal effect was achieved by *L. plantarum*. Around 55% and 54% of total ketone were removed by 5-hour and 10-hour of *L. plantarum* fermentation. The greatest ketone removal achieved by *L. casei* and probiotic strains were only 21% and 33%. The ketone removal was not as effective as the aldehyde removal in all three groups of bacteria strains.

The alcohol production happened along with the LAF process. The LAF treatment produced alcohol in a significant amount. The least amount of alcohol was produced by *L. plantarum,* and samples treated with mix strains of probiotics produced the most amount of alcohol. The relative amount of alcohol became 11 times within 20 hours of probiotics fermentation. For all groups of samples (sample A, sample B and sample C), 5-hour and 10-hour fermented samples do not contain alcoholic aroma. However, the alcoholic smell was observed for a 20-hour

fermented sample. As a result, the 10-hour fermented sample was considered more desirable than a 20-hour fermented sample.



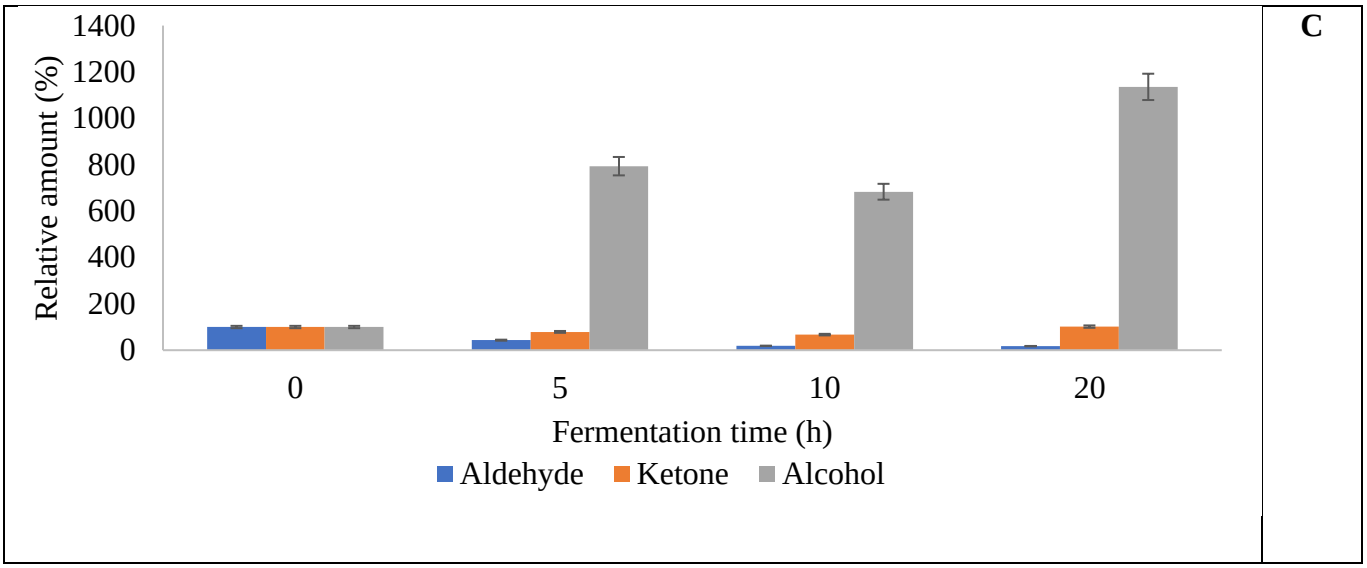



**Figure 4.1.** Total relative amount of volatile compound (aldehyde, ketone, and alcohol) concentration of PPIs, which was fermented with (A) *Lactobacillus plantarum*, (B) *Lactobacillus casei*, and (C) mix strains of probiotics compared to control.

Based on the results of total aldehyde, ketone, and alcohol removal, *L. plantarum* treated sample achieved the most desirable results. It reduced alcohol and ketone content by 75% and 54%, respectively. Even though 133.7% of alcohol was produced, it is unnoticeable. Since the PPIs sample in this study was mainly optimized for yogurt production, and yogurt normally contains around 2% of alcohol. A trace amount of alcohol compound is not detectible by the normal consumer. Moreover, the alcohol being produced can mask other off-flavour compounds presented.

### 4.3.2 Optimization of volatiles profile development during *Lactobacillus plantarum* fermentation

According to the results of **section 4.3.1**, *L. plantarum* achieved the best fermentation results amongst the three groups of bacterial stains, and as a result, detailed unpleasant aroma removal kinetics of *L. plantarum* was studied in the next step. The growth curve of *L. plantarum* in 10% pea protein suspension under an anaerobic environment at 37 ºC is shown in **Figure 4.2.** The exponential growth phase was started before 5 hours of fermentation and ended at around 20 hours of fermentation. In MRS broth the exponential growth phase of *L. plantarum* at 37 ºC under

anaerobic condition was in-between 2 to 8 hours (Smetanková, et al., 2012). As a result, the *L. plantarum* grew slower in PPIs solution than MRS broth.

The results of *L. plantarum* growth were consistent with the pH change of the PPIs sample. The acidity of the PPIs solution after different LAF treatment times is shown in **Figure 4.3**. Since lactic acid has been produced during the LAF, sample pH decreased along with the increase of fermentation time. The pH decreased the most from 5-hour to 20-hour of fermentation and stopped decreasing after 20 hours of fermentation. This is because the bacteria changed from the exponential phase to the stationary phase, and the nutrient in the sample was depleted by the bacteria growth. Acidity change of the PPIs alters aspects of the manufacturing process, such as the product quality, texture, and composition of the products (Smetanková, et al., 2012)

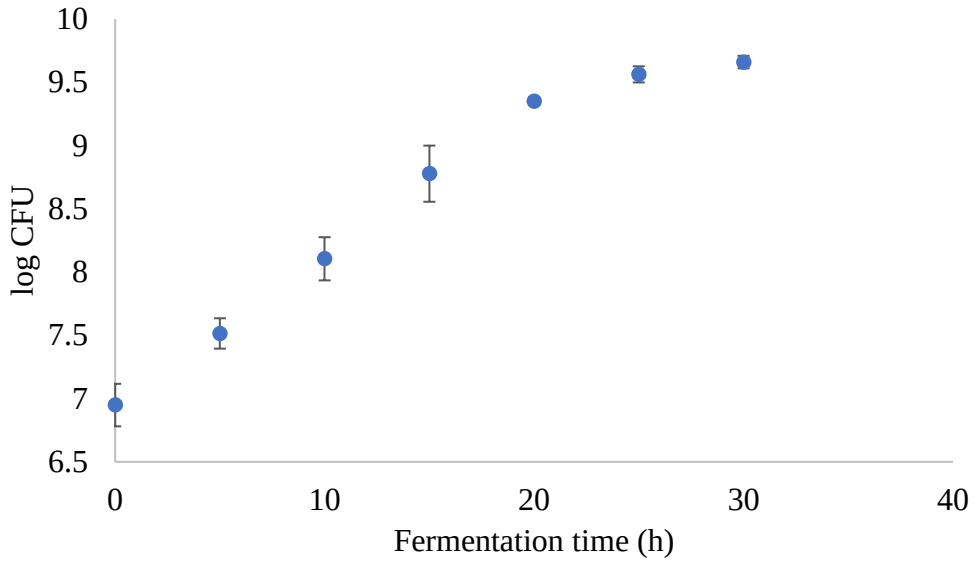



**Figure 4.2.** Growth curve of *Lactobacillus plantarum* fermented PPIs solution (10%) under anerobic conditions at 37ºC.

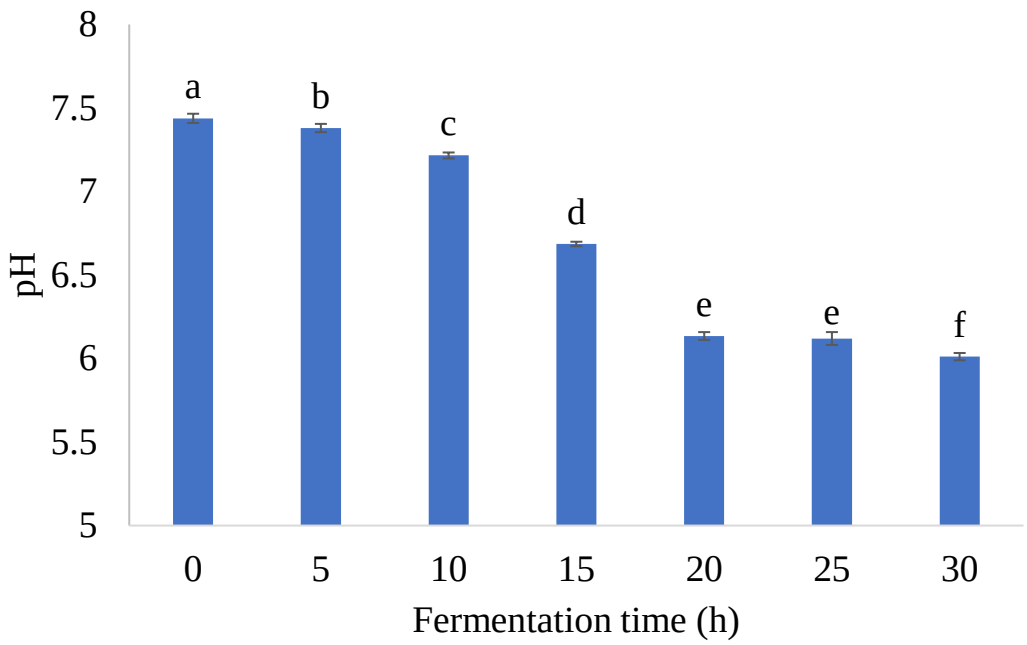



**Figure 4.3.** Decrease of pH values of PPIs solution (10%) fermented with *Lactobacillus plantarum* (anaerobic conditions, 37ºC) in different fermentation times (0, 5, 10, 15, 20, 25, 30 h).

The concentration of aroma compounds belonging to aldehyde, ketone and alcohol group is shown in **Table 4.4.** LAF treatment reduced the concentration of aldehyde significantly. Pentanal and nonanal intensity decreased with the increase of fermentation time, and both compounds were non-detectable after 15 hours of fermentation. Hexanal existed in all fermented and unfermented samples, but the concentration was decreasing during the fermentation process. The concentration of benzaldehyde fluctuated during the fermentation and it was undetectable after 10-hour LAF treatment.

No relationship was observed between ketone compounds concentration and fermentation time. Ketone compounds including acetone and 2-heptanone were produced during the LAF process. However, the concentration of 3,5-octadien-2-one was first reduced and then produced.

Alcohol was produced during the LAF. No alcohol compound was detected in control, however after five hours of fermentation 121 ppb of 1-hexanol and 21 ppb of 1-octen-3-ol were

produced. After 15-hour of LAF, three alcohol compounds were detected including 1-pentanol (30 ppb), 1-hexanol (360 ppb), and 1-octen-3-ol (25 ppb). 1-Pentanol (91 ppb), 1-hexanol (1057 ppb), 1-octen-3-ol (72 ppb) and benzenemethanol (81 ppb) were generated by 30 hours of LAF treatment.

**Figure 4.4** shows the total relative amount of volatile compounds (aldehyde, ketone, and alcohol) concentration of PPIs that were fermented with *Lactobacillus plantarum* in 0, 5, 10, 15, 20 and 25 hours. Aldehyde content was decreased with increase of fermentation time. Total ketone and alcohol concentration were first reduced and then increased along with fermentation. The intersection of best fit lines is around 15 hours of fermentation. The minimum amount of total ketone (51 ppb) and alcohol (93 ppb) were achieved after 10-hour fermentation. No significant difference in ketone content between control and 15-hour fermented sample. However, the aldehyde content in the 15-hour fermented sample (456 ppb) was lower than the 10-hour fermented sample (913 ppb). No significant reduction in aldehyde content was achieved by 10-hour LAF treatment. When considering all three chemical groups of aroma compound (aldehyde, ketone and alcohol), the optimum final aroma profile was achieved after 10 and 15 hours of fermentation. The aroma profile of 10-hour and 15-hour fermented samples were comparable.

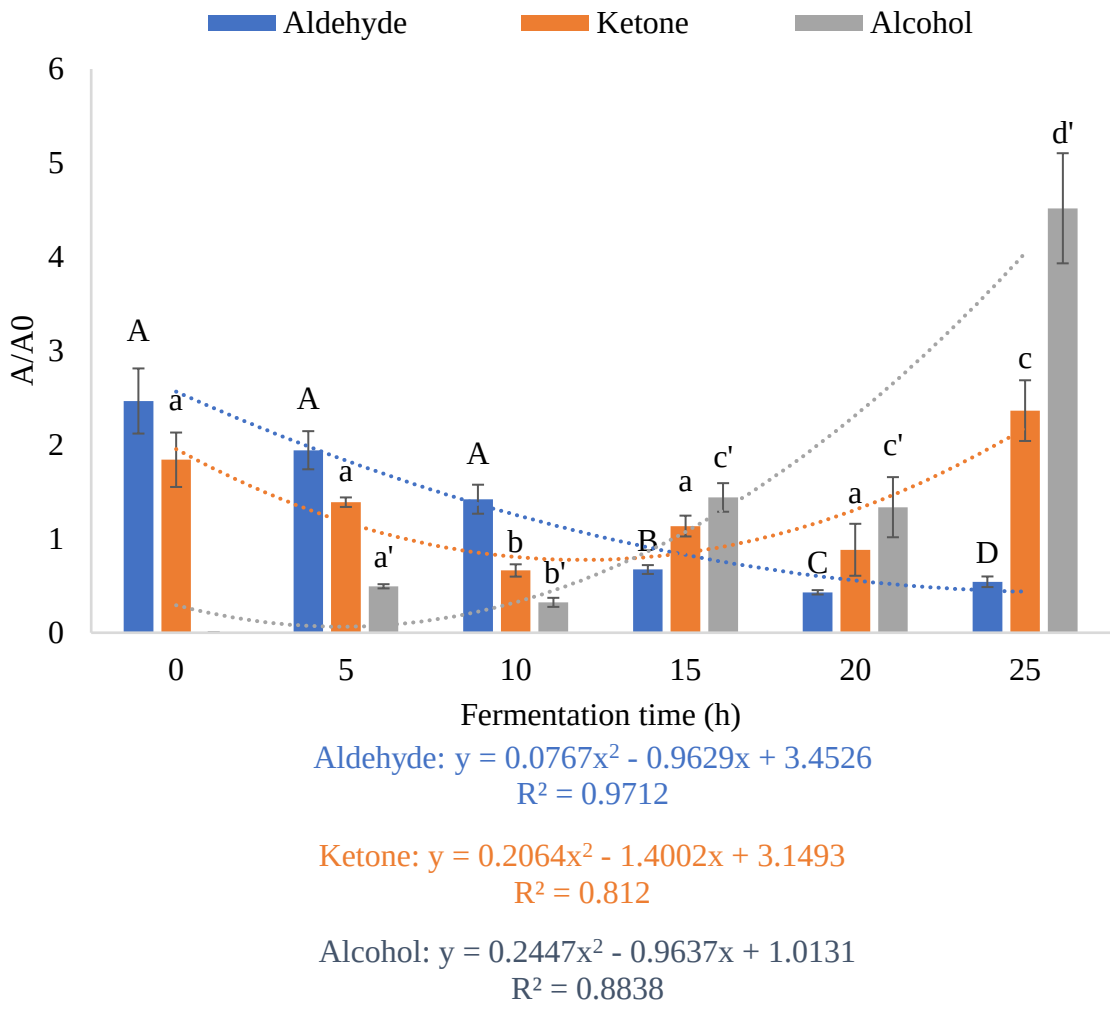



**Figure 4.4.** Total relative amount of volatile compounds (aldehyde, ketone, and alcohol) concentration of PPIs that were fermented with *Lactobacillus plantarum* in 0, 5, 10, 15, 20 and 25 hours. Means with different letters are significantly different (P<0.05) from each other.

**Table 4.4** Aldehyde, ketone and alcohol compounds concentration (**ppb**) detected in *Lactobacillus plantarum* fermented PPIs by using SPME-GC-MS.

| Chemical class | Retention time (min) | Compound identification | Fermentation time (h) | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | **0** | **5** | **10** | **15** | **20** | **25** |
| **Aldehyde** | 4.51 | Pentanal | 90.65 ± 13.48 | 100.30 ± 9.91 | 68.18 ± 1.56 | nd | nd | nd |
| | 6.27 | Hexanal | 1138.00 ± 297.30 | 901.30 ± 179.70 | 743.10 ± 140.60 | 362.50 ± 36.56 | 258.50 ± 19.49 | 281.40 ± 22.14 |
| | 17.35 | Nonanal | 31.82 ± 5.51 | 107.90 ± 7.09 | 102.20 ± 7.59 | nd | nd | nd |
| | 21.32 | Benzaldehyde | 99.19 ± 4.22 | 135.00 ± 13.75 | nd | 93.50 ± 9.36 | 62.35 ± 6.37 | 88.34 ± 77.46 |
| **Ketone** | 3.33 | Acetone | nd | nd | nd | nd | nd | 9.09 ± 2.70 |
| | 9.20 | 2-Heptanone | 25.64 ± 8.10 | 35.61 ± 3.25 | 20.87 ± 0.77 | 31.55 ± 5.12 | 30.25 ± 10.32 | 77.91 ± 10.93 |
| | 17.21 | 2-Nonanone | 6.38 ± 0.62 | 8.21 ± 0.82 | 5.32 ± 0.67 | 6.49 ± 1.06 | 5.96 ± 2.40 | 17.41 ± 3.17 |
| | 21.36 | 2-Decanone | nd | nd | 8.01 ± 3.47 | 7.96 ± 1.50 | 2.75 ± 4.76 | nd |
| | 21.87 | 3,5-Octadien-2-one and isomers | 62.96 ± 19.66 | 48.71 ± 5.58 | 11.11 ± 2.41 | 33.90 ± 5.90 | 25.98 ± 14.54 | 65.73 ±16.62 |
| | 26.25 | Ethanone, 1-(3-butyloxiranyl)- | 10.04 ± 3.65 | 5.00 ± 4.34 | 5.48 ± 0.68 | 7.12 ± 1.18 | 2.72 ± 4.72 | 11.00 ± 10.10 |
| | 21.40 | 3-(Hydroxy-phenyl-methyl)-2,3-dimethyl-octan-4-one | 2.945 ± 0.5100 | 8.944 ± 0.765 | nd | nd | nd | nd |
| **Alcohol** | 11.55 | 1-Pentanol | nd | nd | nd | 29.78 ± 5.671 | 28.04 ± 9.446 | 91.38 ± 18.87 |
| | 15.45 | 1-Hexanol | nd | 121.4 ± 9.339 | 77.04 ± 19.66 | 359.8 ± 65.62 | 335.2 ± 140.1 | 1057 ± 228.0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 19.20 | 1-Octen-3-ol | nd | 20.99 ± 1.806 | 16.07 ± 4.473 | 25.22 ± 4.699 | 21.70 ± 10.18 | 72.30 ± 30.10 |
| 21.36 | Benzenemethanol | nd | nd | nd | nd | nd | 81.21 ± 16.83 |

The results shown are the average of triplicate assessments and are expressed as mean ± standard deviation.
Components are listed on the order of their chemical family.
All compounds were identified by comparison with mass spectra and retention index database.
n.d., not detected, below limit of quantification.

## 4.4 Conclusion

Pea is a nutritious food ingredient that has huge potential to be used in a variety of food products. However, the distinct aroma profile limits the application of pea as a protein source in the food product development. In this study, LAF treatment was applied to PPIs to reduce off-flavour. The volatile compounds in the pea protein sample belonging to aldehyde, ketone and alcohol group were identified and quantified by using SPME-GC-MS method developed in Chapter 3. The performance of L. plantarum, *L. casei,* and mix strains of probiotics was analyzed and compared. The result showed that L. plantarum achieved the best result in eliminating the unpleasant aroma. As a result, the individual performance of L. plantarum in aroma removal was then analyzed. Final aroma profiles of PPIs achieved by 10-hour and 15-hour of *L. plantarum* fermentation were comparable. Around 58% and 36% of original aldehyde and ketone compounds were remaining after the 10 hours of fermentation, and 93 ppb of alcohol compound was produced. After 15-hour of fermentation, around 27% of aldehyde and 62% of ketone were remaining, and 415 ppb of alcohol was produced. These aroma profile changes were considered desirable for the PPIs which will be implemented into the yogurt substitute production.

## Chapter 5: Effect of LAF on functional properties of PPIs

### 5.1 Introduction

Functional properties of a protein are critical quality attributes. It is highly related to the sensorial qualities of the final product. Previous chapter showed that 10-hour *L. plantarum* fermentation was effective in improving the aroma profile of PPIs. In this chapter, the alternations of functional properties caused by the LAF treatment were analyzed. In order to characterize the functional properties of protein isolates, Bradford protein assay was performed to test the water-soluble protein content. Moreover, total and soluble protein fractions are separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Water-holding capacity and oil-holding capacity were tested. In addition, emulsifying properties, including emulsifying activity and emulsifying stability, were measured. Foaming properties, including both foaming capacity and foaming stability, were also determined. Lastly, the density and colour parameters were studied. All parameters were measured for PPIs samples that have been fermented with different times to generate the trend of the functional properties change.

### 5.2 Materials and methods

#### 5.2.1 Materials

In protein analysis, bovine albumin and Coomassie Plus - The better Bradford Assay™ Reagent were purchased from VWR Chemicals (Canada) and Thermo Scientific (Canada), respectively. The 10% precast gel, 10% running buffer and 4x sample loading buffer were purchased form Bio-rad (Canada). Protein ladder was purchased from ABchem (Canada), and EZblue was purchased from Sigma (Canada). Canola oil (Mazola®) used for functional properties analysis was purchased locally (Vancouver, Canada), and the same oil has been used for oil holding capacity and emulsifying properties analysis.

### 5.2.2 Sample preparation

Sample was prepared in the same method as mentioned in section 4.2.2. Ten percent of PPIs solution was inoculated with 7 log CFU/ml of *Lactobacillus plantarum*. After the inoculation, sample was incubated at 37ºC in anaerobic environment for 5, 10, 15, 20, 25 and 30 hours. The fermentation was terminated by freeze the sample at -20 ºC, and then the sample was freeze-dried into dry powder form. The freeze-drying process was only applied to treated sample, the control has not been freeze dried.

### 5.2.3 Protein analysis

#### 5.2.3.1 Water-soluble protein analysis

The water-soluble protein in the sample was tested by the Bradford protein assay. The standard curve was prepared by 0-2000 µl/ml of Bovine Albumin (BSA) solution. The sample for testing was prepared by dissolving 0.2 g of PPIs into 10 ml of dd water. After the vortex and centrifugation of the suspension, the supernatant was taken for the water-soluble protein analysis. Ten µl of each BSA standard and tested samples were added into a 96-well plate, and 200 µl of Coomassie plus reagent was then added into each well. After 10-minute incubation at room temperature, absorbance was measured at 595nm by spectrophotometer (Tecan infinite M200 Pro).

#### 5.2.3.2 SDS-PAGE

SDS-PAGE analysis was performed according to the method of Beuchat (1977). The sample was prepared by dissolving 0.2g of PPIs powder in 10 ml of dd water. The suspension was vortexed and then centrifuged, the supernatant was taken for the analysis. The treated and untreated sample was prepared by dissolving 0.2g of powder in 10 ml of dd water, and the supernatant was used for the analysis. The untreated sample (control) was diluted into two different concentrations, which were 2/3 and 1/3 of the supernatant previously prepared. The dilution was performed in

order to roughly compare the sample concentration by comparing the colour intensity of the protein bands. Samples were electrophoresed on the 10% precast gel (Bio-rad). Prior to electrophoresis, protein hydrolysate was denatured by one-minute microwave heating (Kenmore). Ten μL of 4x Sample loading buffer (Bio-rad) was then added to the 30 μL of the protein solution sample. Five μL of protein ladder (ABchem) was loaded into the first lane, and 20 μL of different PPIs samples were loaded to the next lanes, and the gels were run at 110v for approximately 1.5 hours to complete. The gel was then fixed and stained with EZblue (**Sigma**) for 1 hour. After the staining, the gel was washed with dd water and soaked in dd water overnight in the fume hood. The molecular weights of the polypeptides were estimated by comparing the position of the band with the molecular weight markers.

### 5.2.4 Functional properties

#### 5.2.4.1 Water holding and oil holding properties

The method of water holding capacity and oil holding capacity measurement was modified based on the Kaushik et al. (2013) method. Water or oil holding capacity represents how much water or oil per gram of protein can retain. The experimental procedures were as follows: First, 1g of protein powder was weighed into a pre-weighed centrifuge tube, then 15 ml of water/canola oil was added to the centrifuge tube. Make sure the sample was thoroughly wetted by a vortex. Next, the sample was centrifuged for 10 min at 3000 x g. After the centrifuge process, the supernatant was discarded, and the weight of the wet sample was measured. Water/oil holding capacity was calculated by the sample weight increase (wet sample wt.-Dry sample wt.) divided by the dry sample weight.

#### 5.2.4.2 Emulsifying properties

Emulsifying activity and stability were determined following the method of Neto et al. (2001) with modification. PPIs solution (50 ml, 1%) and 25 ml of canola oil were mixed together to create the emulsion by using a probe sonicator (Ultra turrax® T25 basic) for 15 mins. The emulsion was centrifuged at 1100 x g for 5 min. The height of the emulsified layer and total layer of the sample was then measured. The emulsifying activity was calculated by **Equation 5.1**:

$$\text{EA\%} = \frac{height\ of\ emulsified\ layer\ in\ the\ tube}{height\ of\ total\ content\ in\ the\ tube} \text{ x } 100 \qquad \textbf{Equation 5.1}$$

For the determination of emulsifying stability, the emulsion was prepared by the same method, and the emulsion was treated with an 80ºC water bath for 30 min before the centrifugation. The height of the emulsified layer was measured after centrifugation. The emulsifying stability was calculated as follows (**Equation 5.2**):

$$\text{EA\%} = \frac{height\ of\ emulsified\ layer\ in\ the\ tube\ after\ heating}{height\ of\ emulsified\ layer\ before\ heating}\ X\ 100\% \qquad \textbf{Equation 5.2}$$

**5.2.4.3 Foaming properties**

Foaming capacity and stability were determined following the method of Chawla et al., 2017. PPIs solution (3%) was prepared by dissolving the PPIs powder in dd water and mix with a magnetic stir for 45 min. The PPIs solution was then mixed with a blender (Cuisinart SPB-600FR) for 10 min, and the solution was immediately transferred into a graduated cylinder. The volume was measured right after the whipping (VAW), and the volume of the solution was measured again after 30 min of standing (VAS). The foaming capacity and stability were calculated by **Equation 5.3** and **Equation 5.4,** respectively. VBW is the volume of the solution before whipping.

$$\text{FC (\%)} = \frac{\text{VAW}-\text{VBW}}{\text{VBW}} \text{ X } 100\% \qquad \textbf{Equation 5.3}$$

$$FS\ (\%) = \frac{VAS - VBW}{VBW}\ X\ 100\% \qquad \textbf{Equation 5.4}$$

#### 5.2.4.4 Color and density

The colour profile of the samples was measured by the Hunter lab machine (LabScan XE). Bulk density was measured based on the method of Kaur and Singh (2007) with modification. PPIs sample was milled into the fine powder, and around 20 grams of PPIs sample was weighed and transferred into a graduated cylinder. The exact weight was recorded. The graduated cylinder was gently tapped until there was no diminution of the sample level, and the sample volume was measured. The bulk density was calculated as the mass of the sample per unit volume of the sample (**Equation 5.5**).

$$\rho = \frac{mass\ (g)}{volume\ (cm3)} \qquad \textbf{Equation 5.5}$$

### 5.3 Statistical Data analysis

All extraction and quantification processes were performed in triplicates, and new samples were freshly prepared and used for each run. One-way analysis of variance (ANOVA) and T-test was used to test the significant difference between samples.

### 5.3 Results and discussion

#### 5.3.1 Protein analysis

#### 5.3.1.1 Water-soluble protein analysis

The result of water-soluble protein content is shown in **Figure 5.1**. LAF brought significant impact to the water-soluble protein content of PPIs. Untreated pea protein samples contained 68

mg/g of water-soluble protein. From 0 to 10 hours of fermentation, the water-soluble protein content was continuously decreasing along with the treatment time, and the fermentation treatment reduces the water-soluble protein content to 66% of the control after 10 hours of fermentation treatment. The protein solubility is affected by the solvent pH, and the lowest solubility is achieved at the isoelectric point of the PPIs (Lam et al., 2018). According to the previous studies, the isoelectric point of PPIs is around pH 4-6 irrespective of cultivar and extraction techniques (Boye et al., 2010; Taherian et al., 2011). Based on the pH analysis of PPIs during the fermentation (**Figure 4.3**), the pH of the protein solution was decreased from 7.56 to 6.59. The pH of the protein was reduced throughout the fermentation process and got closed to the isoelectric point. As a result, the solubility was decreased with the increase of the fermentation time. Moreover, LAF changed the protein configuration and lead to the exposure of the hydrophobic group. Hydrophobic groups of the peptide bound together, and the protein solubility decreased as a result. After 15 hours of fermentation, soluble protein content decreased to 21%, and water-soluble protein content of samples that have been fermented longer than 15 hours were similar. The possible reason is that all protein had been precipitated out, and only small water-soluble peptides were remaining. This result is consistent with the SDS-PAGE results shown below.

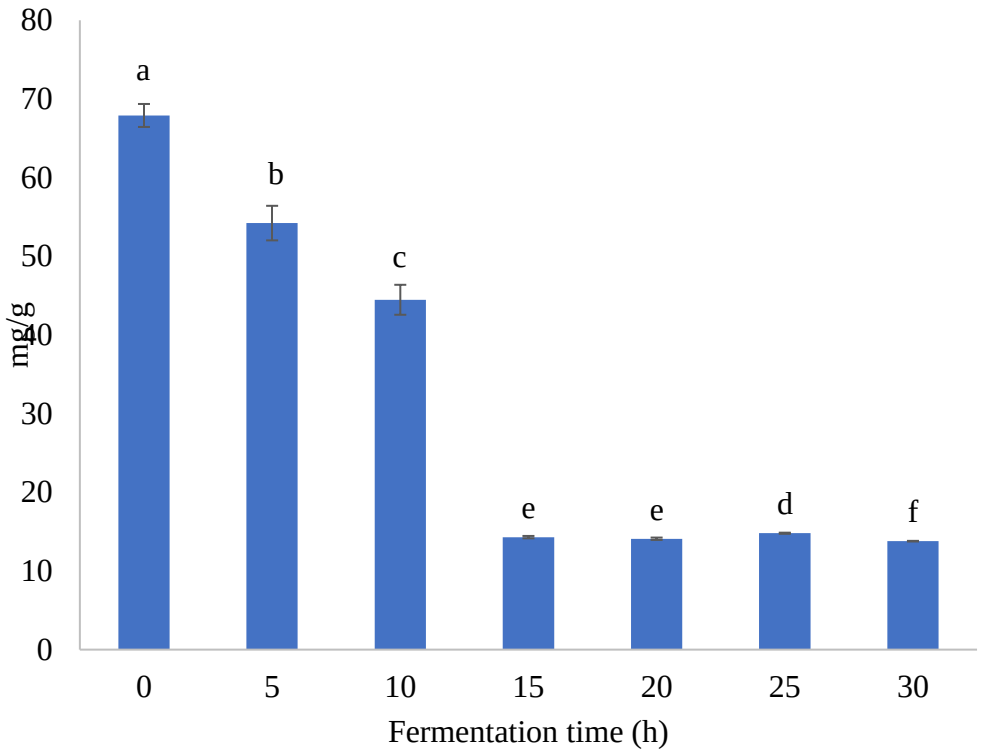



**Figure 5.1.** The water-soluble protein concentration of control (unfermented) and LAF treated PPIs samples at different fermentation times (5, 10, 15, 20, 25, 30 h). Means with different letters are significantly different ($P<0.05$) from each other.

**5.3.1.2 SDS-PAGE**

The sample was prepared by dissolving 0.2g of PPIs powder in 10 ml of dd water. The control was then diluted into 1/3 and 2/3 of this concentration. By comparing the colour intensity of the sample to the control and diluted control, a rough result of protein concentration can be generated. **Figure 5.2** shows that 5-hour and 10-hour LAF treated sample has no clear difference to the control. The change of colour intensity and band position was minor. This means that protein configuration change has not happened. However, after 15-hour of fermentation, protein band with bigger molecular weight no longer existed, and small peptides were remaining. The prolonged LAF treatment caused the protein configuration change and reduced the water solubility of the protein. In the meantime, large proteins have been degraded to smaller peptides. After the insoluble

peptides bind together and precipitate out, small soluble parts were remaining and shown at the bottom part of the SDS-PAGE picture. This protein degradation was assumed to happen between 10 to 15 hours of fermentation, since 15-hour, 20-hour, 25-hour, and 30-hour fermented PPIs samples all shared a similar protein pattern. No further degradation had occurred after the 15-hour of fermentation, and this result was consistent with the result generated by the water-soluble protein analysis (**Section 5.2.1.1**).

The identities of the protein bands could not be confirmed from this study. However, Lipoxygenase (92.7kDa), convicillin (77.9-72.4 kDa), vicilin (44-50 kDa) and $\alpha$-legume (34-39 kDa) usually exist in the pea protein extract, and their band sizes are consistent with the bands shown in control, 5-hour fermented, and 10-hour fermented samples. As a result, the protein that has been degraded during the LAF treatment could be lipoxygenase, convicillin, vicilin and $\alpha$-legume. The smaller protein fragments shown in 15-hour, 20-hour, 25-hour, and 30-hour fermented samples could be albumin (24-29 kDa, β-legumin (20-24 kDa) and other low molecular peptides (Reinkensmeier et al., 2015; Barac et al., 2010).

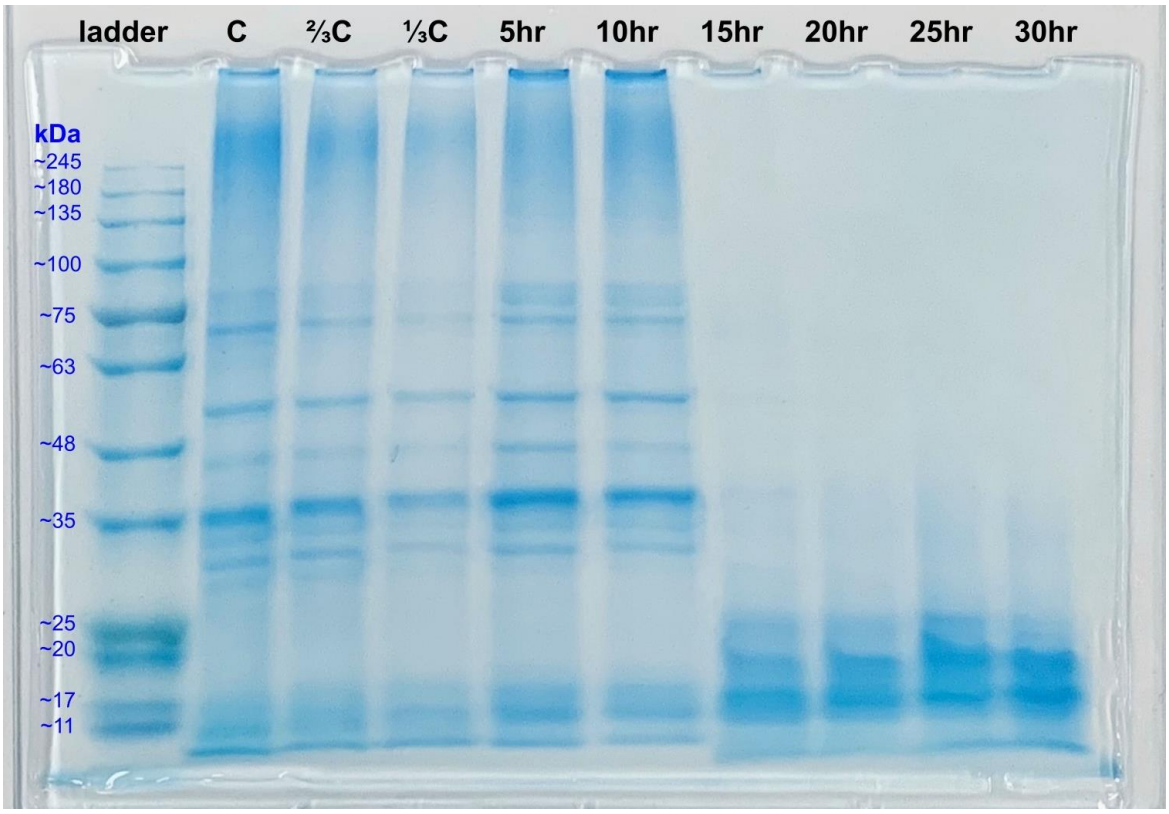



**Figure 5.2.** SDS-PAGE patterns of control and LAF treated PPIs samples at different fermentation times. From left to right, lane 1 for protein markers; lane 2-4 for control in 3 different concentrations, lane 5-10 for LAF treated PPIs, LAF-5 hour, LAF-10 hour, LAF-15 hour, LAF-20 hour, LAF-25 hour, LAF-30 hour, respectively.

### 5.3.2 Effect of LAF on functional properties of PPIs

Functional properties of the protein ingredient play a significant role in food product development and production. It is highly related to the product quality, shelf life and organoleptic characteristic of the food product. PPIs sample in this study is mainly used for non-dairy yogurt production. The ideal product is expected to have a high water/oil holding capacity and emulsifying properties and low foaming capacity.

#### 5.3.2.1 Water binding and oil binding properties

The oil holding capacity of all treated samples was higher than the untreated sample, and the water holding capacity of all treated samples was lower than the control. Based on the result

shown in **Figure 5.3**, the freeze-drying process could lead to lower water-binding capacity but a higher oil binding capacity. Research by Mirhosseini and Amid (2013) showed that the freeze-drying process increased the oil-holding capacity of durian seed gum.

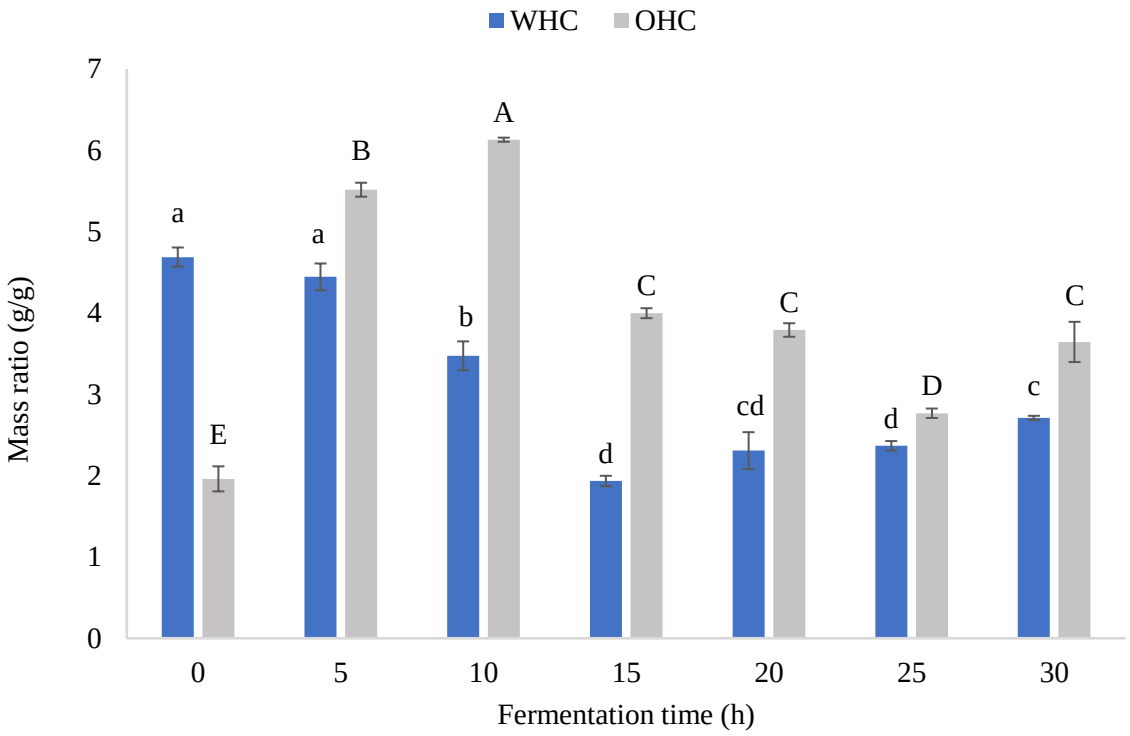



**Figure 5.3.** Water-holding and oil-binding capacity of unfermented (0 h) and fermented PPIs samples. Data are presented as mean ± standard error (n=3). Means within different letters are significantly different (P<0.05) from each other.

From 0-10 hours of fermentation, the water holding capacity of the sample decreased with the fermentation time, and the oil holding capacity increased with the fermentation time. This is LAF changed the protein folding and increased the exposure of the hydrophobic region. LAF treatment led to the protein configuration change, and more hydrophobic components were exposed out. As a result, LAF treated samples generated a lower water-holding capacity but a higher oil-holding capacity (Caballero et al., 2003).

Starting from 15 hours of fermentation, the water holding capacity increased with the fermentation time, and oil holding capacity decreased with the fermentation time. Previous protein analysis results (**Figure 5.1** and **Figure 5.2**) show that the protein solubility decreased starting from 15 hours of fermentation, and the only small peptide was remaining in the solution. All other protein content was insoluble. The insoluble protein has a lower water holding capacity than the soluble protein, so the sample which has been fermented longer (15-30 hours) have lower water holding capacity than the sample have been fermented shorter (5-10 hours) or unfermented (0-hour). Moreover, **Figure 5.2** also shows that the protein configuration change happened after 15 hours of fermentation. The protein matrix structure, especially pore size significantly affects the water/oil holding capacity of the protein (Lam et al., 2018).

The water holding was achieved by coeffects of ion-dipole, dipole-dipole, dipole-induced dipole and hydrophobic interactions, and protein has the lowest water holding capacity at its isoelectric point (Lam et al., 2018). At the isoelectric point, protein-protein interaction is at the greatest, and electrostatic attraction between protein and water is at the least (Lam et al., 2018). As shown in **Figure 4.3**, the pH of the PPIs was decreasing through out the fermentation process and getting close to the isoelectric point of the PPIs, which is 4-6 (Estevinho & Rocha, 2018). As a result, the increase of the matrix acidity decreased the water holding capacity.

The optimum result of the aroma profile was obtained after 10 hours of fermentation. The 10-hour fermented PPIs sample generated a higher oil holdings capacity and lower water holdings capacity compared to the control. A higher oil holding capacity was considered as a desirable change. This is because coconut cream has been added into the yogurt formula in order to increase the creaminess mouthfeel of the product. PPIs sample has a higher oil holding capacity that would bind better with the coconut cream. During the food processing, a lower water holding capacity

can lead to liquid loss which affect the texture of the final product in an undesirable way (Lam et al., 2018). When implement the final product into the yogurt production, a lower water holding capacity could lead to a syneresis effect of the final yogurt. Whether this change is acceptable for a specific product can only be decided when the sample is made, and the shelf-life test is performed.

#### 5.3.2.2 Emulsifying properties

Emulsifying properties include emulsion activity and emulsion stability. Emulsion activity represents the capability of the protein solution to be emulsified with oil, and the emulsion stability stands for the ability of the emulsion to stay unchanged. **Figure 5.4** showed that the emulsifying properties of treated samples have no significant difference with the control.

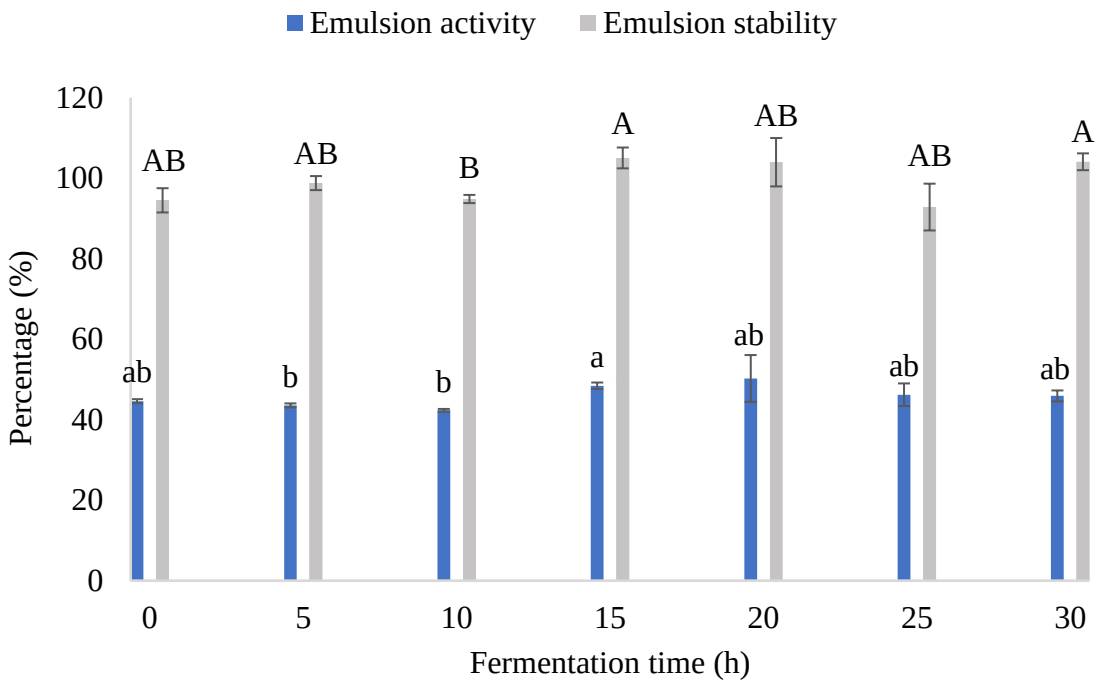



**Figure 5.4.** Emulsifying properties of unfermented (0 h) and fermented PPIs samples. Means with different letters are significantly different (P<0.05) from each other.

Emulsifying properties is highly related to the hydrophobicity of the protein, since protein bind with water and oil faction at the same time and serve as a bridge. Previous water/holding capacity (**section 5.2.2.1**) and protein analysis **(section 5.2.1)** results showed that the protein

configuration and hydrophobicity have changed. The result of unchanged emulsifying properties could be a combined effect of many changes. According to the pH analysis of the protein solution (**Figure 4.3**), the acidity of the protein was increased with the increase of fermentation time. Moreover, the protein shows a higher emulsifying property when the pH is closer to the isoelectric point (Caballero et al., 2003). As a result, the sample has been fermented longer should have better emulsifying properties, since the pH of the protein solution was closer to the isoelectric point of the pea, which is 4-6 (Estevinho & Rocha, 2018).

In addition, the results of **section 5.2.1** showed that protein had been degraded into smaller peptides. The smaller peptides can easily migrate into the oil-water interface and exhibit a better emulsifying property (Chawla et al., 2017). However, previous water-soluble protein analysis (**section 5.2.1)** showed that the water-soluble protein concentration has decreased. A lower solubility will lead to smaller emulsifying properties (Caballero et al., 2003).

Last but not least, alcohol was produced by LAF treatment. Alcohol could also affect the emulsifying properties by change the hydrophobicity of the protein. In summary, the changes in different parameters lead to the shift of emulsifying properties in different directions. However, no significant difference existed in emulsifying properties for LAF treated sample and control.

#### 5.3.2.3 Foaming properties

Overall, more prolonged LAF treatment samples generated higher foaming properties than the less treated sample. By the appearance, the less fermented samples formed small uniform foam. In contrast, samples that have been fermented longer tended to form “bubble-like” rigid foam. This is because the prolonged LAF treated sample had a lower water solubility, and a more rigid protection coat was formed for the air bubbles and prevent the bubbles from collapsing (Lam et al., 2018).

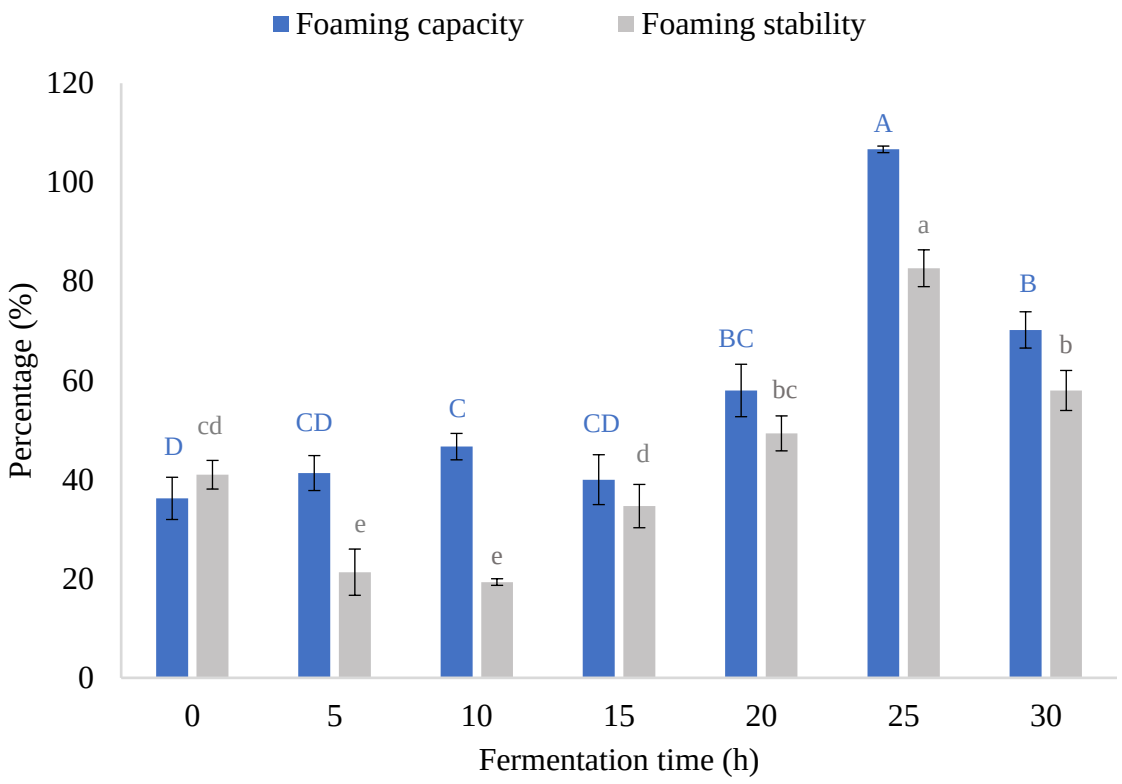



**Figure 5.5.** Foaming properties (right) of unfermented (0 h) and fermented PPIs samples. Means with different letters are significantly different (P<0.05) from each other.

Similar to emulsifying properties, the foaming properties was decided by the ability of protein to migrate into water-air interface. Protein realign its hydrophobic groups towards the air, and the hydrophilic group towards the water (Çabuk et al., 2018). As a result, the foaming properties are related to the pH of the sample. The foaming stability was at the highest at the isoelectric point of the protein since minimal electrostatic repulsion was existed (Lam et al., 2018). In this study, the longer fermented sample generated a higher acidity (**Figure 4.3**), which was closer to the isoelectric point of the pea (4-6) (Estevinho & Rocha, 2018). Hence, the foaming properties was increasing with the increase of fermentation time. Çabuk et al. (2018) also showed that the foaming capacity of *L. plantarum* fermented pea protein enriched flour was significantly affected by fermentation time, pH, and their associated interactions. Moreover, the foaming

stability of *L. plantarum* fermented pea protein enriched flour was affected by fermentation time and the interaction between fermentation time and pH. In addition, hydrophobicity of the PPIs could positively affect the foaming capacity. This is because, the ability of protein to create the interfacial area decides the foaming capacity (Lam et al., 2018).

In this study, the LAF treated sample was used for dairy yogurt production, and a smaller foaming effect is considered a desirable change to avoid the bubbly product. Based on the result of aroma analysis and protein analysis, the 10-hour fermented sample achieved the best result. After the 10-hour LAF treatment, no significant difference was observed in foaming capacity, and lower foaming stability was noticed. The change in foaming properties after 10 hours of treatment was considered ideal.

#### 5.3.2.4 Color and density

**Figure 5.6** shows the appearance of samples that have been fermented with 0 to 30 hours from left to right. From less fermented and longer fermented samples, the colour is turning darker and yellower, and the product is turning form a powder form into a sandy form. The conclusion generated by observation was consistent with the results generated by the colour analysis and density analysis, which are discussed below.

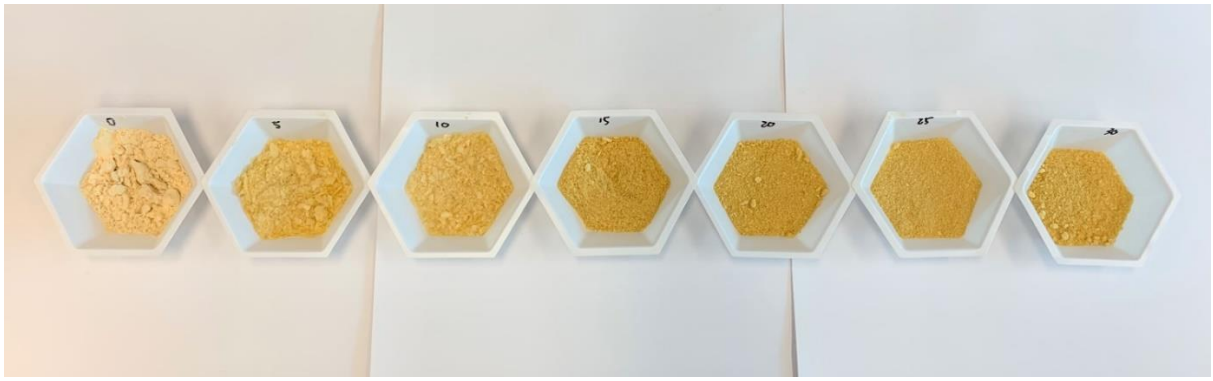


**Figure 5.6.** The appearance of the PPIs sample, from left to right are untreated (control), 5-hour, 10-hour, 15-hour, 20-hour, 25-hour and 30-hour LAF treated samples.

**Figure 5.7** represents the colour result generated by hunter lab. The 0 point in the y-axis stands for the control (unfermented), ΔL represents the lightness change, and it is negative for all treated samples, which means that the LAF treatment has decreased the lightness of the sample. The positive Δa and Δb value showed that the LAF treated sample gave the PPIs sample a redder and yellower appearance. ΔE represents the overall colour change. There is no clear trend of colour change in relation to the fermentation time. This is because the colour pigment might be degraded and formed throughout the fermentation process. The alcohol content and acidity were increased by LAF treatment. The change to the protein matrix can also affect the colour shown.

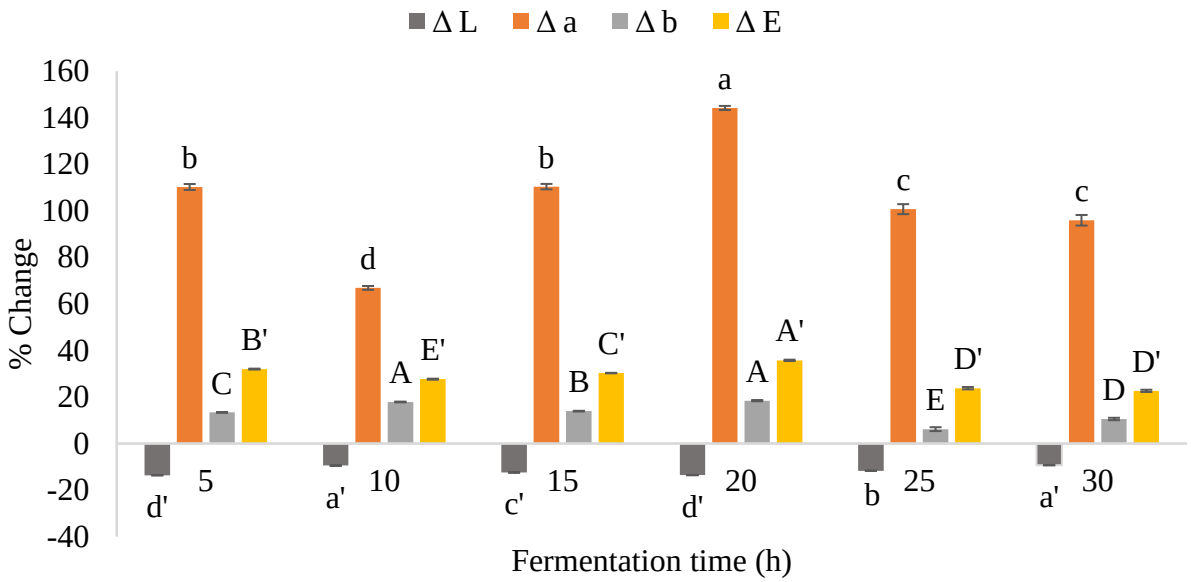



**Figure 5.7.** Percentage of ΔL, Δa, Δb, ΔE change of control and LAF treated PPIs samples at different fermentation times (5, 10, 15, 20, 25, 30 h) respectively. Means with different letters are significantly different ($P<0.05$) from each other.

The density results are shown in **Figure 5.8**. The longer fermented sample (15-hour, 20-hour, 25-hour, and 30-hour) have higher density values than the less fermented samples (5-hour and 10-hour). The control showed the highest density because the control has not been freeze-dried treated, and the powder is finer than the treated sample.

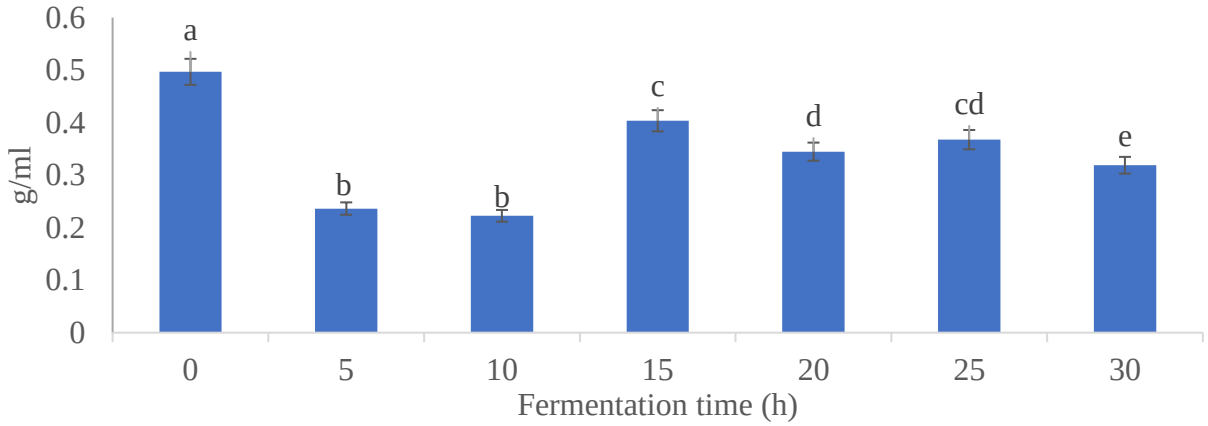



**Figure 5.8.** Bulk density of unfermented (0 h) and fermented PPIs samples. Data are presented as mean ± standard error (n=3). Means with different lowercase letters are significantly different ($P<0.05$) from each other.

### 5.4 Conclusion

In this chapter, the functional properties of the LAF treated PPIs were studied. Results showed that prolonged LAF achieved significant changes to the protein configuration, water-soluble protein content, water and oil holding capacities, foaming capacities, colour, and density. No significant change was found in emulsifying properties, including emulsifying activity and emulsifying stability. From chapter 4, The 10-hour *L. plantarum* fermented PPIs were found to have the best final overall aroma profile. As a result, the functional properties of the 10-hour LAF sample was analyzed and compared to the control. The result showed that 10-hour LAF treatment did not change the protein configuration, and the water-soluble protein has decreased by 35%. Oil-holding capacity and water-holding capacity of 10-hour LAF sample have increased by 200% and reduced by 26%, respectively. Moreover, no significant difference was observed for the emulsifying properties. In addition, foaming capacity has increased by 29%, and foaming stability was decreased by 53%. Whether these changes to the functional properties are acceptable or not will be decided after the sensory test, and shelf-life test was performed.

## Chapter 6: Sensory analysis of lactic acid fermented PPIs

## 6.1 Introduction

The main purpose of this study is to understand the difference and similarities between samples and their corresponding attribute intensities. As a result, the method of sensory testing is one of the most convincing methods to test the effectiveness of various treatments. Moreover, sensory analysis can be used to support the results that have been generated from the previous chapters 4 and 5. In this chapter, a descriptive analysis was performed to identify and understand the differences and similarities of all sensory patterns, including the appearance, aroma, taste, texture and aftertaste of PPIs that have been treated by different methods.

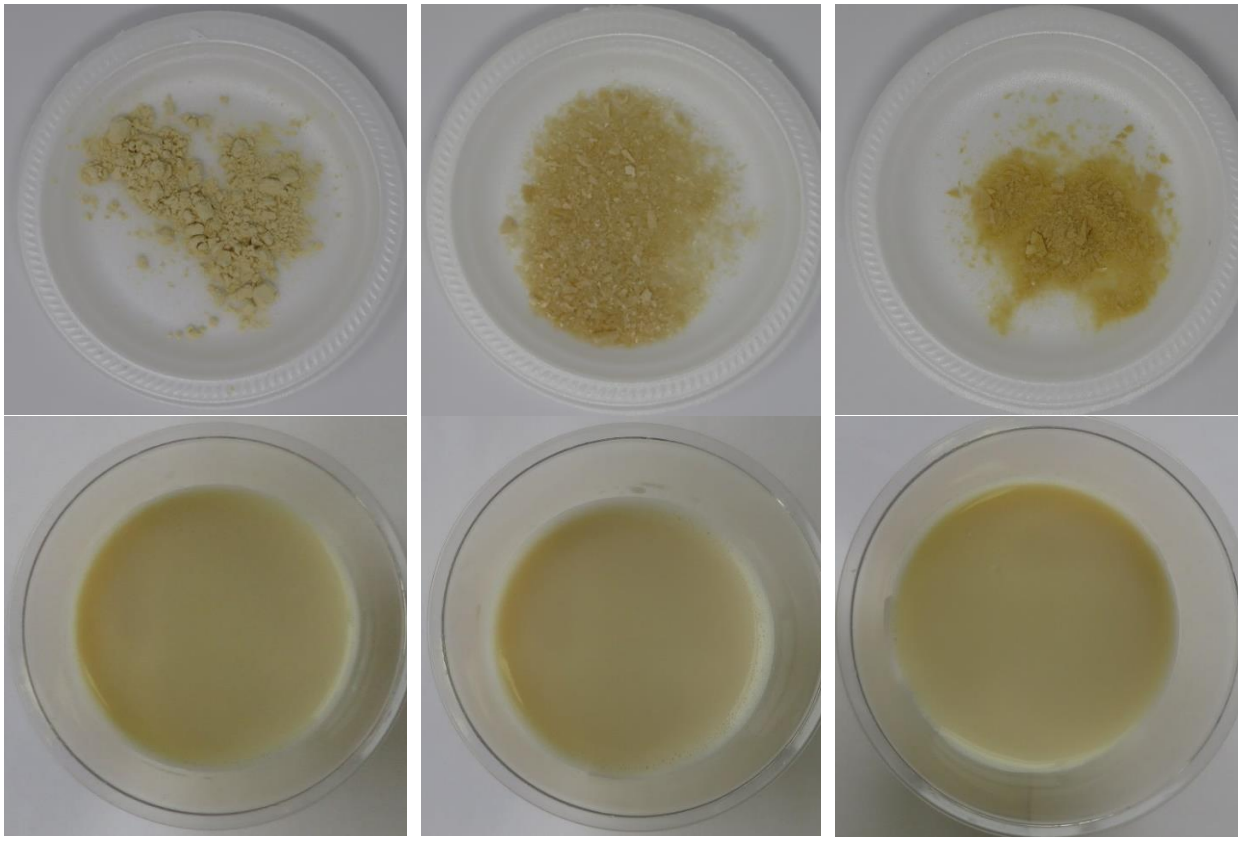
## 6.2 Materials and methods

A descriptive analysis test of protein samples was performed by trained panellists at ACCE facility (Canada) to validate the sensory modalities of PPIs that were treated by different treatment methods. The flavour profile of four kinds of PPIs samples, including the control, DSI treated sample, 10-hour *L. plantarum* fermented sample, and VMD treated sample, has been studied. The VMD treatment was not the focus of the study, so the result of the VMD treated sample was only shown in appendices. The panellists were trained for sensory testing of pea protein with appropriate references.

The difference and similarities for all sensory modalities, including appearance, aroma, texture, and aftertaste, have been analyzed among different pea protein samples. Each attribute was rated on the 15-point scale, where 0 represents none, and 15 represents very strong. Detailed attributes and their definition are shown in **Table B1** (appendices). This scale includes the ability to use a tenth of a point, so the sample has the potential to be differentiated by 150 scale units.

Randomized and blind samples were provided to panellists at room temperature. Samples were evaluated in two sessions, and four products were evaluated in each session. As a result, a

total of 2 replications were performed for each product. Session 1 was performed on February 6th, 2020, at 12 pm, and session two was performed on February 7th, 2020, at 9:30 am. For the detailed sample preparation and schedule for the descriptive sensory test, please refer to **Table B2** in appendices. Pea protein solution (120 ml) was served in clear 7 oz plastic cups with lids (**Figure 6.1**). All samples were swallowed by panellists, and attributes including aroma, flavour, texture were rated by panellists individually. Sample appearance was analyzed by all panellists together in consensus.



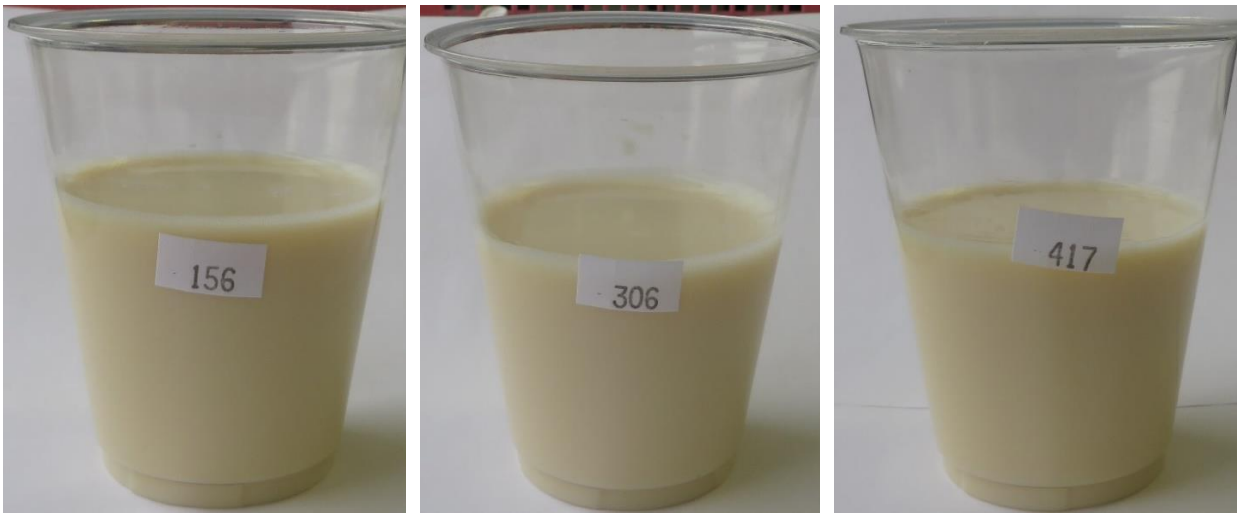



**Figure 6.1.** The appearance of the different PPIs samples in dry powder and solution. From left to right are the control, DSI treated sample, and LAF treated samples.

### 6.3 Results and discussion

Appearance, aroma, flavour and mouthfeel/texture difference between three different samples were analyzed. A summary of the result was presented below in **Table 6.1.** The detailed result will be explained in section **6.3.1-6.3.3.** For detailed data and Fisher's least significant difference (LSD) test results, please refer to **Table C3-C7** in appendices.

**Table 6.1.** Summary table of the descriptive analysis result.

| Modality | Control | DSI | LAF |
|---|---|---|---|
| Appearance | Lowest amount of foam | Highest amount of foam | Very low amount of foam |
| | Darkest in colour with highest colour intensity (4.8) | Lightest in colour with lowest colour intensity (3.0) | Low medium colour intensity |
| | Very low chroma (2.5) | Lowest chroma (2.0) | Lowest chroma (2.0) |
| Aroma | Low medium overall aroma intensity | Lower overall aroma intensity | Lower overall aroma intensity |
| | | Lowest green/grassy notes | |

| | | | |
|---|---|---|---|
| | Most noticeable green/grassy aroma | | Lowest beany/vegetative aroma |
| Flavour | Low medium overall flavour intensity | Low medium overall flavour intensity | Lowest overall flavour intensity |
| | Slight amount of bitterness | Lower amount of bitterness | Lower amount of bitterness |
| | Highest level of raw/beany, beany/vegetative, and green/grassy flavours | Lowest amount of green/grassy, chalky, and metallic flavours | Lower amount of beany (raw & vegetative), and chalky flavours |
| Texture | Very low number of particles, but slightly more than DSI | Lowest number of particles and amount of astringency, but differences are minute and not likely to be detected by consumers | Very low number of particles |

### 6.3.1 Appearance

The descriptive analysis result of sample appearance is shown in **Figure 6.2**. The least amount of foam (score 1.3) and was observed in control. This observance was consistent with the result generated by the foaming capacity analysis (section 5.2.2.3). The lowest foaming capacity was generated in the untreated sample (control). LAF appeared to be closer to the control than DSI treated sample in the amount of foam. The control contains the highest chroma (score 2.5) among all samples in solution. Both DSI and LAF treatment reduced the chroma level of PPIs sample, and the chroma result of the LAF treated sample and the DSI were the same (score 2.0). This finding was consistent with the results generated by previous colour analysis (section 5.3.2.4). The colour analysis showed that LAF treatment had decreased the PPIs in lightness. The highest colour intensity was observed in control (score 4,8), and the least colour intensity was shown in the DSI

treated sample (score 3.0). Both LAF and DSI treatments reduced the colour intensity, but the LAF treated sample (score 3.7) was appeared to be closer to control than DSI treated sample.

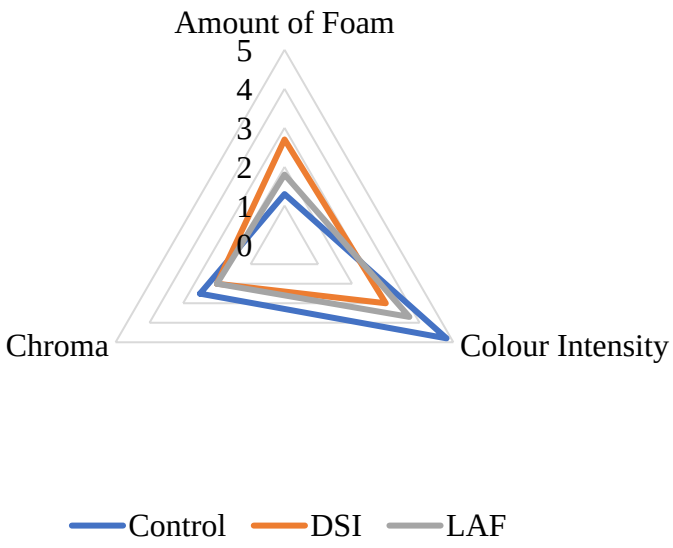



**Figure 6.2.** Descriptive analysis result of sample appearance.

DSI treated samples had the greatest difference in appearance compared to control. DSI treated samples generated the highest amount of foam and lowest colour intensity in three groups of samples. This means that the DSI treated sample obtained a lighter and duller yellow colour than untreated (control) and LAF treated samples. Moreover, less amount of bubbles was formed by LAF treatment. As a result, LAF treatment generated a better sample appearance than DSI treatment.

All products were found to produce more foam in the 2$^{nd}$ rep, which may be a result of sample variability. In conclusion, the LAF treatment delivered a better result than the DSI treatment.

### 6.3.2 Aroma

**Figure 6.3** shows the descriptive analysis result of sample aroma. Both aroma strength and aroma profiles were evaluated for aroma analysis. The results showed that the control had the strongest aroma profile, which delivered green and grassy smells. These types of off-flavour were mainly caused by n-hexanal and n-hexanol, which are formed by the oxidation of plant lipids (Tangyu et al., 2019).

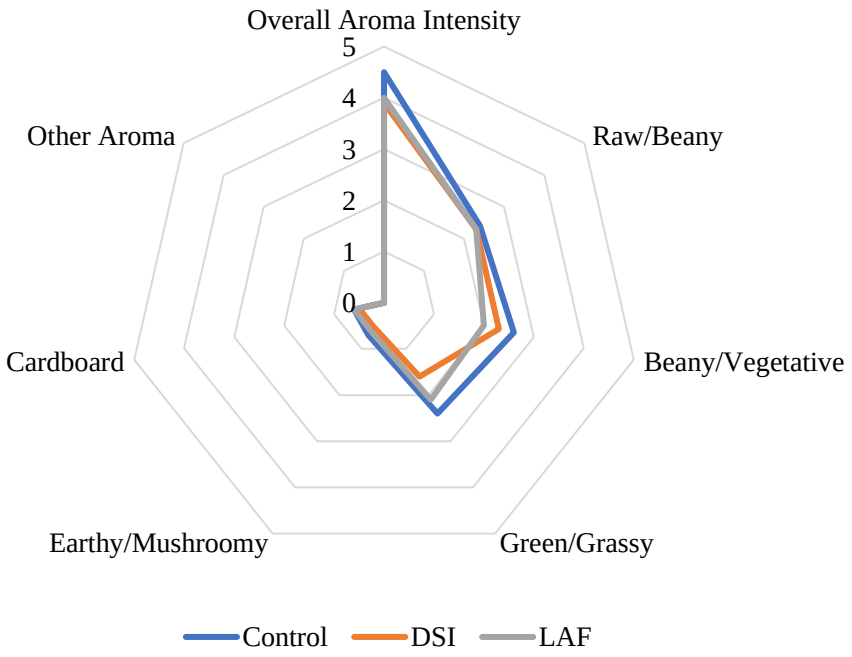



**Figure 6.3.** Descriptive analysis result of sample aroma.

DSI and LAF treated samples were very similar in aroma profile, and both contained a lower aroma intensity than control. No significant difference was observed for the overall aroma intensity between DSI (score 3.9), and LAF (score 4.0) treated samples. Based on the result of the descriptive analysis, both LAF and DSI treatment was the same effective in eliminating the unpleasant aroma from the control. LAF (score 2.0) had the least amount of beany/vegetative aroma. The highest amount of green/grassy aroma was found in control (score 2.4), while the

lowest was observed in DSI treated sample (score 1.6). The tiny amount of cardboard aroma was found in all samples, ranging from score 0.5-0.8, and no significant difference existed between all samples in raw/beany, earthy/mushroomy aroma. No other aroma was notified in all samples other than what have listed.

By comparing the LAF treated sample with control, LAF treatment significantly reduced the aroma attributes including overall aroma intensity, beany/vegetative, and green/grassy aroma. This result was consistent with the result generated by Schindler et al. (2012), fermented PPIs generated a more desirable odour. Moreover, LAF did not adversely alter the pleasant aroma of PPIs (Schindler et al., 2012). In other plant-based protein, LAF was found associated with the production of desirable volatile flavours; Acetaldehyde (peanut and fruity aroma) was produced in soy fermentation (Horáčková et al., 2015). Diacetyl (2,3-butanedione) which delivers a butterscotch smell was produced during the cereal-based fermentation (Peyer et al., 2016).

### 6.3.3. Flavour

The earthy and beany flavour in plant protein were considered undesirable, especially in the countries without the tradition consumption of plant protein product (Tangyu et al., 2019). The objectionable tastes in PPIs are mainly originated from phenols (e.g tannis and saponins) terpenes, glucosinolates, and flavonoids. (Drewnowski & Gomez-Carneros, 2000). In this study, off-flavour characteristics of PPIs samples included raw, beany, vegetative, grassy, chalky and bitterness were analyzed (**Figure 6.4**). Control (score 4.1) delivered the most off-flavour in three different samples, and LAF treated sample (score 3.7) had the lowest overall flavour intensity. Mital and Steinkraus (1979) also showed that LAF improve the flavour of plant-based milk substitute to make it more acceptable to western taste. No significant difference was found between samples in attributes including Sweet, Earthy/Mushroom, and Cardboard. Control had the highest bitterness (score 1.9),

raw/beany flavour (score 2.5), beany/vegetative flavour (score 2.3), green/grassy (score 2.0), and chalky (score 2.7). DSI treated sample contained the least amount of chalky (score 2.2) and metallic (score 0.3) flavour. LAF treated sample had the smallest overall flavour intensity and bitterness.

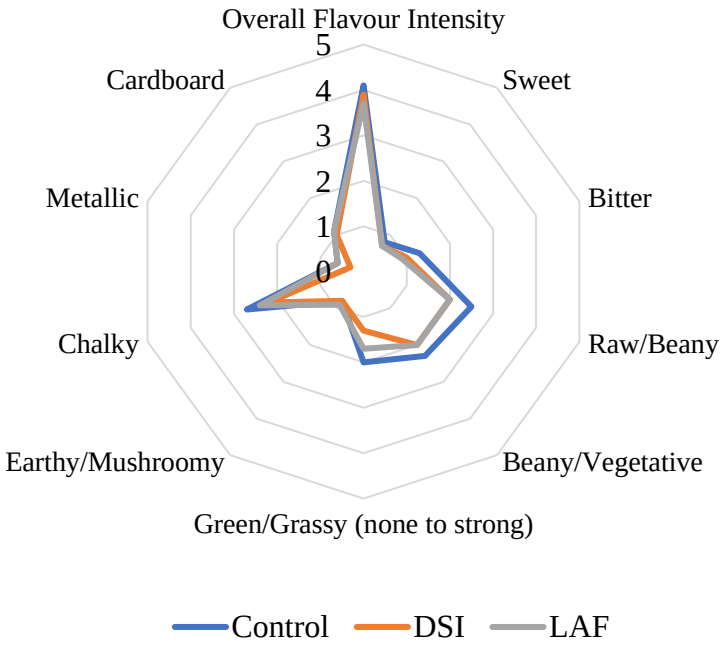



**Figure 6.4.** Descriptive analysis result of sample flavour.

### 6.3.4. Texture and aftertaste

In this study, attributes including thickness/viscosity, astringency/mouth-drying, fatty/greasy mouth coat, and amount of particles were analyzed (**Figure 6.5**). No significant difference was noted among the Thickness/Viscosity or Fatty/Greasy mouthfeel. However, DSI has the least amount of particles (score 0.6) and astringency (score 2.3) texture than other samples, and control has the greatest amount of particles (score 0.8) and astringency (score 2.5) texture. This is because the mouthfeel of particles and astringency were mainly due to the insolubility of protein. The consumer purchase willingness was negatively affected by chalky, sandy, and thin

mouthfeel exist in PPIs due to the presence of insoluble particles (Peyer et al., 2016). DSI sample was treated with elevated temperature during the steam injection, and this higher temperature increased the protein solubility in water. LAF (score 0.7) treated sample has no significant difference with the other two samples in particle texture. It has a significantly higher astringency texture than DSI treated sample.

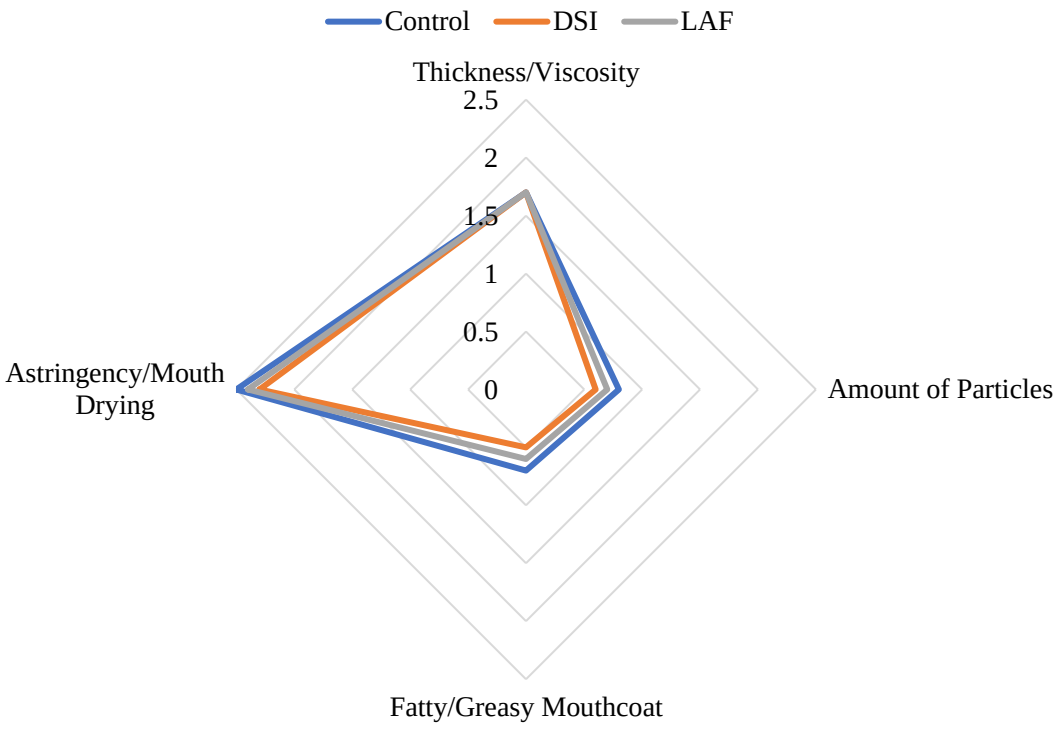



**Figure 6.5.** Descriptive analysis result of sample texture.

The descriptive analysis of sample aftertaste is shown in **Figure 6.6**. Small texture differences were noticed by the panellists in the aftertaste. Both LAF and DSI treatment lowered the astringency/mouth-drying aftertaste (score 2,1). The control showed the highest sensation of astringency (score 2.3) among three different samples in the aftertaste. The difference between the three products for aftertaste is minor and not noticeable by the average consumer. According to

Matsuura et al., (1989), some bioactive compounds including isoflavonids were associated with unpleasant aftertaste.

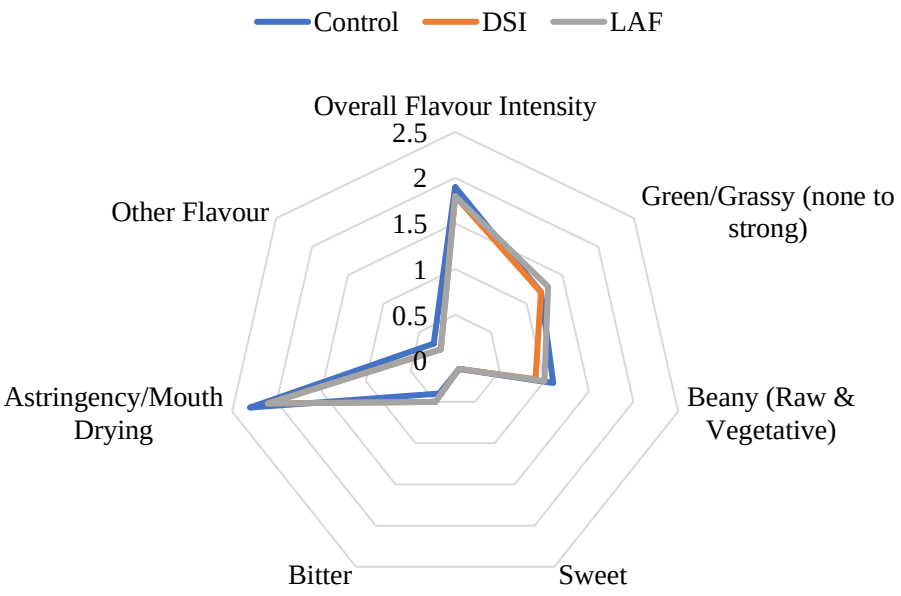



**Figure 6.6.** Descriptive analysis result of sample aftertaste.

## 6.4 Conclusion

According to the descriptive sensory analysis result, both LAF and DSI treatments were effective in improving the sensorial qualities of PPIs. Sensory attributes, including aroma, flavour, and texture, and aftertaste, were improved after the treatments. DSI treatment achieved a better result in lowering the sample texture. No significant difference was observed between control and LAF treated samples in texture. However, LAF treated sample achieved better results than DSI treated sample in appearance, overall flavour intensity, overall aroma intensity and aftertaste.

# Chapter 7: Conclusion and future directions

## 7.1 Conclusions

Plant protein is cost-effective and environmentally friendly. However, the distinct “hay” and “green” like aroma limits the application of pea in the food industry. In this study, a simple water extraction method was implemented to recover volatiles that is known to contribute to off-flavour from three different plant proteins. Headspace: solid-phase micro-extraction, coupled with gas chromatography-mass spectrometry, enabled the identification of volatile indicator compounds and relative contribution to the total volatiles present. This method will be useful in comparing the efficacy of different food processing methods used to remove off-flavour, associated with these volatile aromas.

LAF was found to be an effective method to reduces the off-flavour from plant protein. The effectiveness of LAF on off-flavour removal by *L. casei*, *L. plantarum,* and mix strains of probiotics were compared by comparing the aroma profile of treated samples. *L. plantarum* achieved the most desirable aroma profile result among three groups of bacteria, and the detail performance of *L. plantarum* fermentation in eliminating unwanted volatile compounds was analyzed. Ten-hour fermentation by *L. plantarum* achieved the best overall product quality in both flavour and functional properties. The change to the water-soluble protein and protein configuration was minor. Aldehyde and ketone content was reduced by 42.38% and 64.03%, respectively, and a small amount of alcohol (93.11 ppb) was produced. The aroma profile change was considered desirable for the PPIs, which will be used for pea yogurt production.

A descriptive analysis test has performed at the end of the study to compare the flavour of LAF treated, DSI treated and untreated pea protein sample. The results showed that positive results

had been achieved by LAF treatment. The 10-hour *L. plantarum* fermentation improved the overall quality of the pea protein isolates in appearance, aroma, flavour, texture and aftertaste.

### 7.2 Future research directions

The next step of this study is to implement the 10-hour *L. plantarum* fermented PPIs as the main ingredient to produce the pea-based dairy substitute. The consumer acceptance sensory test and the shelf life test will be performed on this newly formulated dairy substitute. If the new dairy alternative has high market acceptability and normal shelf stability, the *treatment* method would be scaled up to the industry level.

The result of this study showed that *L. plantarum* fermentation successfully improved the flavour profile while maintained the functional properties of PPIs. However, the functionality of *L. plantarum* fermentation on other kinds of plant-based protein has not been studied. As a result, the effect of *L. plantarum* fermentation on different plant-based proteins such as soy and brown rice will be studied. The plant protein which can achieve the best result in the final flavour profile with *L. plantarum* fermentation result will find out. Moreover, different fermented plant-based protein can be implemented in varieties of novel food products based on their flavour characteristics.

In addition, the functionality of varieties of lactic acid-producing bacteria stains in improving the aroma profile of PPIs will be explored. It is also valuable to study the co-effect of two or more lactic acid-producing bacteria stains, or the lactic acid-producing bacteria with yeast strains on off-flavour elimination.

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## Appendices

### Appendix A. Nutritional data and amino acid profile of untreated PPIs sample

**Table A1.** Nutritional data (Typical value for 100g of product)

| Chemical Analysis | Values |
|---|---|
| **Calories (Kcal)** | 390 |
| **Moisture (g)** | 6.0 |
| **Protein (dry matter basis) (g)** | 80.0 |
| **Total Fat (g)** | 6.0 |
| **Saturated Fat (g)** | 1.0 |
| **Mono-unsaturated fat (g)** | 2.0 |
| **Poly-unsaturated fat (g)** | 3.0 |
| **Cholesterol** | 0.0 |
| **Trans Fatty Acids (g)** | 0.0 |
| **Carbohydrates (g)** | 4.0 |
| **Sugars (g)** | 0.0 |
| **Dietary fibre (g)** | 4.0 |
| **Soluble fibre (g)** | 0.0 |
| **Insoluble fibre (g)** | 4.0 |
| **Ash (g)** | 5.0 |
| **Sodium (mg)** | 750 |
| **Phosphorus (mg)** | 1100 |
| **Potassium (mg)** | 200 |
| **Calcium (mg)** | 400 |
| **Iron (mg)** | 10 |
| **Vitamin D (mg)** | 0.0 |

The information is obtained from Daiya Foods Inc., Vancouver

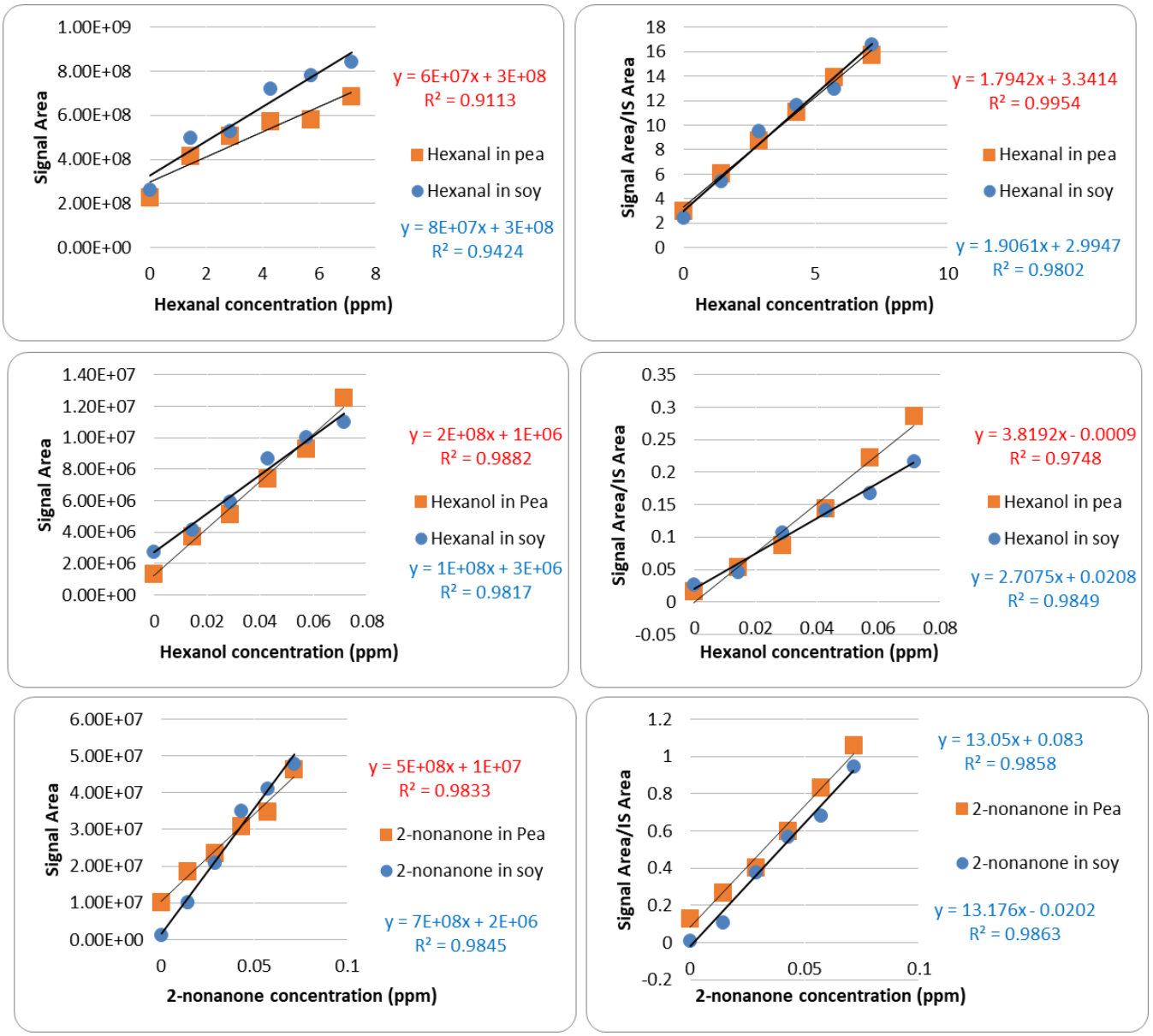
**Table A2.** Amino Acid Profile (Typical data g/100g)

| Essential Amino Acids | Values | Non-Essential Amino Acids | Values |
|---|---|---|---|
| **Arginine** | 6.84 g | Alanine | 3.44 g |
| **Histidine** | 1.99 g | Aspartic Acid | 9.42 g |
| **Isoleucine** | 4.05 g | Cysteine | 0.85 g |
| **Leucine** | 6.80 g | Glutamic Acid | 13.32 g |
| **Lysine** | 6.11 g | Glycine | 3.28 g |
| **Methionine** | 0.85 g | Serine | 3.88 g |
| **Phenylalanine** | 3.86 g | Tyrosine | 3.86 g |
| **Threonine** | 3.05 g | Proline | 3.55 g |
| **Valine** | 4.28 g | | |
| **Tryptophan** | 0.79 g | | |

The information is obtained from Daiya Foods Inc., Vancouver

**Appendix B. The adaptability test of the standard curve on different plant-based protein.**

**Figure B1**: Calibration curves generated for hexanal, 1-hexanol and 2-nonanone in pea and soy protein isolates (calibration curves in red are for pea, and that in blue are for soy).



## Appendix C. The descriptive sensory analysis

**Table C1.** Descriptive analysis attributes and definitions

| | Attribute | Scheme | Definition |
|---|---|---|---|
| **Appearance** | Amount of Foam | None to lots<br>0-15 | The amount of foam or bubbles visible throughout the surface of the product |
| | Colour Intensity | Light yellow to dark yellow<br>0-15 | The intensity or strength of the colour from light to dark |
| | Chroma | Dull to pure/bright<br>0-15 | The degree to the colour is bright/pure (absence of grey) throughout the sample |
| **Aroma** | Overall Aroma Intensity | None to strong<br>0-15 | The total intensity of all aromas perceived in the sample |
| | Raw/Beany | None to strong<br>0-15 | Aromatic associated with unprocessed and/or uncooked legumes |
| | Beany/Vegetative | None to strong<br>0-15 | The degree to which the product smells like cooked beans or green vegetables |
| | Green/Grassy | None to strong<br>0-15 | Green, slightly sweet aromatic associated with cut grass |
| | Goaty/Caproic Acid | None to strong<br>0-15 | Aromatics associated with caproic acid, goat, and gamey |
| | Hay | None to strong<br>0-15 | Aromatic associated with sweet, dry grasses (hay, straw) |
| | Earthy/Mushroomy | None to strong<br>0-15 | Aromatic characteristic of damp soil or mushrooms |
| | Nutty | None to strong<br>0-15 | Aromatic associated with nuts or nut meats |
| | Chalky | None to strong<br>0-15 | Aroma associated with mineral salts such as chalk |

| | | | | |
|---|---|---|---|---|
| | Sweet Aromatics | None to strong 0-15 | Aromatics associated with the impression of a sweet substance |
| | Soap | None to strong 0-15 | The intensity of unscented soap aroma |
| | Fishy/Marine | None to strong 0-15 | Aromatic associated with fish, seaweed |
| | Chemical | None to strong 0-15 | The intensity of chemical odour; iodine, quinine, solvents, and other chemicals |
| | Medicinal | None to strong 0-15 | Aromatic characteristic of band-aids, disinfectant |
| | Metallic | None to strong 0-15 | Aromatics associated with metals, tin cans, or iron |
| | Painty | None to strong 0-15 | Aromatic associated with oxidized oil, linseed oil, and oil-based paint |
| | Cardboard | Not to very 0-15 | Aromatic characteristic of wet cardboard packaging |
| **Flavour** | Overall Flavour Intensity | None to strong 0-15 | The total intensity of all flavours in the sample, including basic tastes |
| | Raw/Beany | None to strong 0-15 | Aromatic associated with unprocessed and/or uncooked legumes |
| | Beany/Vegetative | None to strong 0-15 | The degree to which the product tastes like cooked beans or green vegetables |
| | Green/Grassy | None to strong 0-15 | Green, slightly sweet aromatic associated with cut grass |
| | Hay | None to strong 0-15 | Aromatic associated with sweet, dry grasses (hay, straw) |
| | Earthy/Mushroomy | None to strong 0-15 | Aromatic characteristic of damp soil or mushrooms |

| | | | |
|---|---|---|---|
| | Nutty | None to strong<br>0-15 | Aromatic associated with nuts or nut meats |
| | Chalky | None to strong<br>0-15 | Flavour associated with mineral salts such as chalk |
| | Sweet Aromatics | None to strong<br>0-15 | Aromatics associated with the impression of a sweet substance |
| | Soapy | None to strong<br>0-15 | The intensity of unscented soap flavour |
| | Fishy/Marine | None to strong<br>0-15 | Aromatic associated with fish, seaweed |
| | Chemical | None to strong<br>0-15 | The intensity of chemical flavour; iodine, quinine, solvents, and other chemicals |
| | Metallic | None to strong<br>0-15 | Aromatics associated with metals, tin cans, or iron |
| | Painty | None to strong<br>0-15 | Aromatic associated with oxidized oil, linseed oil, and oil-based paint |
| | Cardboard | Not to very<br>0-15 | Aromatic characteristic of wet cardboard packaging |
| **Basic taste** | Sweet | Not to very<br>0-10 | The taste stimulated by sucrose and other sugars, such as fructose, glucose etc., and by other sweet substances i.e. Artificial sweeteners |
| | Salt | Not to very<br>0-15 | The taste stimulated by sodium salts, such as sodium chloride and sodium glutamate, and in part by other salts, such as potassium chloride |
| | Sour | Not to very<br>0-15 | The taste stimulated by acids, such as citric, malic, phosphoric, etc. |

| | | | |
|---|---|---|---|
| | Bitter | Not to very<br>0-15 | The taste stimulated by substances such as quinine, and caffeine |
| **Texture** | Thickness/Viscosity | Thin to thick<br>0-15 | The degree to which the product feels thick or dense in the mouth as opposed to thin and watery |
| | Amount of Particles | None to many<br>0-15 | The amount of particles felt throughout the sample |
| | Size of Particles | Small to large<br>0-15 | The size of the particles felt throughout the sample |
| | Fatty/Greasy Mouthcoat | None to high<br>0-15 | The amount of fatty/greasy residue felt by the tongue when moved over the surfaces of the mouth |
| | Astringency/Mouth Drying | None to strong<br>0-15 | The shrinking or puckering of the tongue surface caused by substances such as<br>tannins or alum e.g. green banana peel, strong black tea |
| **Aftertaste (after 30 seconds)** | Overall Intensity | None to strong<br>0-15 | The total intensity of all flavours |
| | Green/Grassy | None to strong<br>0-15 | Green, slightly sweet aromatic associated with cut grass |
| | Beany/Raw & Vegetative | None to strong<br>0-15 | The overall intensity of raw/beany and beany/vegetative flavours |
| | Sweet | Not to very<br>0-15 | The taste on the tongue stimulated by sucrose and high potency sweeteners |
| | Sour | Not to very<br>0-15 | The taste on the tongue stimulated by acid, such as citric, malic, phosphoric, etc. |
| | Bitter | Not to very<br>0-15 | The taste on the tongue associated with caffeine and other bitter substances, such as quinine and hop bitters. |
| | Astringency/Mouth Drying | None to strong<br>0-15 | The shrinking or puckering of the tongue surface caused by substances such as |

tannins or alum e.g. green banana peel, strong black tea

**Table C2.** Descriptive analysis sample preparation plan and schedule

| Product | Product # | Rep1 Code | Rep2 Code |
|---|---|---|---|
| **Control** | 1 | 156 | 407 |
| **DSI** | 2 | 306 | 149 |
| **LAF** | 3 | 417 | 920 |

| | Sample Presentation Blinding Code Order | | | Sample Presentation Order | | |
|---|---|---|---|---|---|---|
| **Session 1: Thursday PM (Rep 1)** | Sample 1 | Sample 2 | Sample 3 | Sample 1 | Sample 2 | Sample 3 |
| | 306 | 417 | 156 | 2 | 3 | 1 |
| **Session 2: Friday AM (Rep 2)** | Sample 4 | Sample 5 | Sample 6 | Sample 4 | Sample 5 | Sample 6 |
| | 407 | 149 | 920 | 1 | 2 | 3 |

**Table C3.** Descriptive analysis result for sample appearance.

| ATTRIBUTE (0-15) | Control | DSI | LAF | VMD | LSD | Prob |
|---|---|---|---|---|---|---|
| **Amount of Foam** (none to lots) | 1.3 d | **2.7 a** | 1.8 b | 1.5 c | 0.0 | **<.0001** |
| **Color Intensity** (light yellow to dark yellow) | **4.8 a** | 3.0 d | 3.7 c | 4.3 b | 0.0 | **<.0001** |
| **Chroma** (dull to bright/pure) | **2.5 a** | 2.0 b | 2.0 b | **2.5 a** | 0.0 | **<.0001** |

**Table C4.** Descriptive analysis result for sample aroma

| ATTRIBUTE (0-15) | Control | DSI | LAF | VMD | LSD | Prob |
|---|---|---|---|---|---|---|
| **Overall Aroma Intensity** (none to strong) | 4.5 b | 3.9 c | 4.0 c | **4.8 a** | 0.1 | **<.0001** |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Raw/Beany** (none to strong) | *2.4* | *a* | *2.3* | *a* | *2.3* | *a* | *2.2* | *a* | *0.2* | *0.2038* |
| **Beany/Vegetative** (none to strong) | 2.6 | b | 2.3 | c | 2.0 | d | **2.9** | **a** | 0.2 | **<.0001** |
| **Green/Grassy** (none to strong) | **2.4** | **a** | 1.6 | c | 2.1 | b | 2.0 | b | 0.2 | **<.0001** |
| **Earthy/Mushroomy** (none to strong) | *0.7* | *a* | *0.5* | *a* | *0.6* | *a* | *0.8* | *a* | *0.2* | *0.0527* |
| **Cardboard** (none to strong) | 0.6 | b | 0.5 | b | **0.6** | **ab** | **0.8** | **a** | 0.2 | **0.0265** |
| **Other Aroma** (none to strong) | 0.0 | b | 0.0 | b | 0.0 | b | **0.5** | **a** | 0.0 | **<.0001** |

**Table C5.** Descriptive analysis result for sample flavour

| **ATTRIBUTE (0-15)** | **Control** | | **DSI** | | **LAF** | | **VMD** | | **LSD** | **Prob** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Overall Flavour Intensity** (none to strong) | 4.1 | a | 3.9 | b | 3.7 | c | 4.1 | ab | 0.2 | 0.0001 |
| **Sweet** (not to very) | 0.8 | a | 0.7 | a | 0.7 | a | 0.9 | a | 0.2 | 0.5466 |
| **Bitter** (not to very) | 1.3 | a | 1.0 | b | 0.9 | b | 1.2 | ab | 0.3 | 0.0212 |
| **Raw/Beany** (none to strong) | 2.5 | a | 2.0 | b | 2.0 | b | 2.1 | b | 0.1 | <.0001 |
| **Beany/Vegetative** (none to strong) | 2.3 | a | 2.0 | b | 2.0 | b | 2.3 | a | 0.2 | 0.0036 |
| **Green/Grassy** (none to strong) | 2.0 | a | 1.3 | d | 1.7 | b | 1.5 | c | 0.1 | <.0001 |
| **Earthy/Mushroomy** (none to strong) | 0.8 | a | 0.8 | a | 0.9 | a | 0.9 | a | 0.2 | 0.5247 |
| **Chalky** (none to strong) | 2.7 | b | 2.2 | d | 2.4 | c | 2.9 | a | 0.2 | <.0001 |
| **Metallic** (none to strong) | 0.6 | a | 0.3 | b | 0.6 | a | 0.6 | a | 0.2 | 0.0016 |
| **Cardboard** (none to strong) | 1.1 | a | 1.0 | a | 1.1 | a | 1.1 | a | 0.2 | 0.5502 |

**Table C6.** Descriptive analysis result for sample texture

| **ATTRIBUTE (0-15)** | **Control** | **DSI** | **LAF** | **VMD** | **LSD** | **Prob** |
|---|---|---|---|---|---|---|

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Thickness/Viscosity** (thin to thick) | 1.7 | a | 1.7 | a | 1.7 | a | 1.6 | a | 0.2 | 0.4552 |
| **Amount of Particles** (none to many) | 0.8 | a | 0.6 | b | 0.7 | ab | 0.8 | a | 0.2 | 0.0254 |
| **Fatty/Greasy Mouthcoat** (none to high) | 0.7 | a | 0.5 | a | 0.6 | a | 0.6 | a | 0.1 | 0.0974 |
| **Astringency/Mouth Drying** (none to strong) | 2.5 | a | 2.3 | b | 2.4 | a | 2.6 | a | 0.2 | 0.0031 |

**Table C7.** Descriptive analysis result for sample aftertaste

| **ATTRIBUTE (0-15)** | **Control** | | **DSI** | | **LAF** | | **VMD** | | **LSD** | **Prob** |
|---|---|---|---|---|---|---|---|---|---|---|
| **Overall Flavour Intensity** (none to strong) | 1.9 | a | 1.8 | a | 1.8 | a | 1.9 | a | 0.1 | 0.2008 |
| **Green/Grassy** (none to strong) | 1.2 | a | 1.2 | a | 1.3 | a | 1.2 | a | 0.1 | 0.3860 |
| **Beany** (Raw & Vegetative) (none to strong) | 1.1 | a | 0.9 | a | 1.0 | a | 1.1 | a | 0.2 | 0.1032 |
| **Sweet** (not to very) | 0.1 | a | 0.1 | a | 0.1 | a | 0.1 | a | 0.1 | 0.6562 |
| **Bitter** (not to very) | 0.4 | a | 0.5 | a | 0.5 | a | 0.5 | a | 0.2 | 0.8462 |
| **Astringency /Mouth Drying** (none to strong) | **2.3** | **a** | 2.1 | bc | 2.1 | c | **2.3** | **ab** | 0.2 | **0.0164** |
| **Other Flavor** (none to strong) | 0.3 | a | 0.2 | a | 0.2 | a | 0.3 | a | 0.1 | 0.3521 |