This thesis is dedicated to my parents

Debbie and Michael Marinangeli

for their undying support

"Happiness . . . is not a fish that you can catch"

- Raine Maida -

EFFECTS OF WHOLE AND FRACTIONATED YELLOW PEA FLOURS ON INDICES OF CARDIOVASCULAR DISEASE, DIABETES AND THERMOGENESIS AS WELL AS THE GASTROINTESTINAL MICROBIOME

By

Christopher P.F. Marinangeli

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ABSTRACT

Whole yellow pea flour (WPF) and fractionated yellow pea flour (FPF) are novel functional food ingredients that vary in nutritional composition. Consequently, the health benefits of WPF and FPF remain undefined. The purpose of this research was to identify the effects of WPF and FPF on risk factors and morbidities associated with cardiovascular disease, diabetes and obesity as well as the gastrointestinal microbiome. Using USDA recommended dosages of WPF and FPF, clinical endpoints and the colonic microbiome were investigated using a human clinical trial engaging a cross-over design and a diet and energy controlled paradigm. Humans were also utilized to investigate post-prandial glycemic responses and sensory characteristics of novel functional foods formulated with WPF. Finally, Golden Syrian hamsters were used to assess the impact of high doses of WPF and FPF on clinical endpoints and caecal microbial abundance. Results reveal that USDA recommended dosages of WPF and FPF in humans decreased ($p<0.05$) fasting insulin and estimates of insulin resistance compared to white wheat flour (WF). Android-to-gynoid fat ratios in women were lower ($p=0.027$) in the WPF group compared to the WF group. FPF decreased ($p<0.05$) post-prandial energy expenditure alongside a tendency ($p<0.075$) to reduce carbohydrate oxidation. Novel biscotti and banana bread formulated with WPF induced low post-prandial glycemic responses which were similar to boiled whole yellow peas and significantly lower ($p<0.05$) than white bread. Sensory analysis of novel WPF biscotti and banana bread demonstrated that WPF-based food products are palatable and acceptable for human consumption. Golden Syrian hamsters consuming diets containing 10% WPF and FPF induced similar reductions ($p<0.05$) in fasting insulin levels compared to controls. However, animals consuming WPF increased ($p<0.05$) oxygen consumption while FPF decreased ($p<0.05$) fasting glucose levels. In addition, terminal restriction fragment length polymorphism analysis revealed that WPF and FPF induced distinct shifts in caecal microbial populations within the phyla Firmicutes. Finally, pyrosequencing analysis of human fecal microbiota demonstrated that FPF and WPF induced shifts in bacterial genera, primarily within Bacteroidetes and Firmicutes. In conclusion, whole and fractionated yellow pea flours are functional food ingredients and can be utilized to manage risk factors for lifestyle-related diseases in humans.

CHAPTER I

INTRODUCTION

Pulses, including yellow peas, lentils, chickpeas, and dry beans, are defined as leguminous crops yielding up to 12 grains or seeds enclosed in a pod. The term "pulses" is reserved for those crops that are harvested solely as a dry grain, thus excluding green beans, green peas and soybeans which are harvested as vegetable and oil crops, respectively. After Wheat, barley, canola and corn, pulses are Canada's the fifth largest crop-based commodity, taking ≈10% of the market share, followed by: oats (≈6%), soybeans (≈4%), rye (≈4%), flax (1%), mixed grains and other special crops (<1%) (*Canada: Grains and Oilseeds Outlook: 2009-10*, 2009; *Pulses and Special Crops (P&SC) Outlook December 15, 2009*, 2009). However, compared to soy, flax and mixed grains, only modest levels of funding and interest have been focused on delineating specific health benefits of pulse crops. Consequently, in 2006 Pulse Canada put forth a major funding initiative to delineate the health benefits of pulse consumption. Pulse Canada invested in this health-focused research to bring value to pulse crops as commodities and foster new approaches for nutritionists, food scientists and industry to urge Canadians to increase their consumption of pulses.

Touted as "super-foods," pulse crops are high in fibre, protein, and antioxidants, while low in fat (Champ, 2002; Leterme, 2002). However, just as all fruits and vegetables are not equivalent in providing specific health benefits; it is likely that different pulse crops produce diverse effects on health outcomes. Consumption of pulses, mainly lentils, chickpeas and beans, have been shown to improve morbidities and risk factors associated with cardiovascular disease and diabetes via modulating circulating lipid levels (Anderson & Major, 2002), post-prandial glucose response (Nestel et al., 2004), post-prandial insulin response (Nestel et al., 2004) and insulin sensitivity (Yang et al., 2006). However, clinical trials have yet to substantiate the effects of pulse crops on body composition, post-prandial energy expenditure, substrate/macronutrient utilization,

hepatic lipid metabolism and the gastrointestinal microbial populations. Moreover, most studies evaluating the efficacy of pulses on health outcomes fail to control for diet and energy intake. Hence, it is unknown whether it is the pulse crop itself or secondary factors that contribute to the effect of pulses on health endpoints. For example, it is unclear whether reduced circulating lipids are secondary to a direct effect of pulse crop-derived bioactives on hepatic lipid metabolism and lipid excretion or the effects of pulse-derived fibres on satiety and subsequent weight loss. Thus, clinical trials that assess the health benefits of pulse crops under a diet and energetically controlled paradigm are required to determine which clinical endpoints pulse crops target directly.

Most studies examining the relationship between pulse crops and disease have utilized lentils, beans, and chickpeas. Albeit a handful of studies investigating the physiological effects of supplemental pea fibre, very little research has examined the health benefits of pea consumption. Currently, peas constitute over 70% of all pulses produced in Canada, with ≈85% of harvested crop being exported to other countries (*Pulses and Special Crops (P&SC) Outlook December 15, 2009*, 2009). By defining the effects of peas on risk factors and morbidities for disease, the value for domestically grown peas should increase, promoting their usage in the development of healthy novel food products for human consumption in North America. Moreover, the fact that yellow peas are routinely separated into two distinct fractions, the hull and cotyledon, questions concerning the effects of each pea constituent on health outcomes remain unanswered. By identifying the health benefits of each fraction, specific components of yellow peas can be isolated and/or concentrated for incorporation into novel functional foods which will positively modulate biological risk factors and morbidities associated with life-style related diseases.

Currently, the USDA recommends that ½ cup pulses/d be consumed as part of a healthy diet (USDA.gov, 2009). One-half cup of dry whole yellow peas represents approximately 50 g. For studies evaluating the efficacy of yellow peas on health outcomes, USDA recommended dosages represent a good launching point for human clinical trials. Depending on the observation that yellow peas produce significant changes and trends toward improvements in clinical endpoints, dosages can be subsequently adjusted for future studies to determine optimal levels of intake.

Palatability is a major obstacle impeding pulse consumption in North America (Desrochers & Brauer, 2001). Pulse crops are described as having a "bean-like" flavor and odour that can be unfavorable. Currently whole and fractionated yellow peas are being utilized to produce novel flours. Pea-based flours present new opportunities for incorporating pulse crops into novel functional foods, such as baked goods, whereby unfavorable sensory characteristics could be masked with additional ingredients. Nonetheless, use of flours as a vehicle to facilitate pulse consumption in humans could impede the ability for pulses to modulate health outcomes. Milling increases the surface area exposure of flour particles to thermal stressors during the cooking process which could alter or breakdown bioactive components (Bishnoi et al., 1994; Bjorck et al., 1994; Hawkins & Johnson, 2005; Jenkins et al., 1982; Wong et al., 1985). Hence, novel functional flours that incorporate whole and/or fractionated yellow peas must be assessed for efficacy in human clinical trials.

The focus of this body of research is to delineate the health benefits of novel whole and fractionated yellow pea-based flours in humans, with a specific focus on cardiovascular disease, diabetes and obesity as well as the gastrointestinal microbiome. Never before have whole and fractionated pulse crops been compared simultaneously in human clinical trials and animal research. Results are anticipated to facilitate and justify the production of novel functional foods that incorporate pulse crops or their components as primary ingredients, bringing value to Canada's pulse industry and better health to Canadians.

THESIS OBJECTIVES

The objectives of the present research was to:

1. Produce novel, efficacious functional foods using whole and fractionated yellow pea flours.

2. Determine the effects of whole and fractionated yellow pea flours on risk factors and morbidities for cardiovascular disease and diabetes including circulating lipids, post-prandial glucose meal-response, fasting insulin, insulin sensitivity and body composition.

3. Determine the effect of whole and fractionated yellow pea flours on post-prandial energy expenditure, thermic effect of food, substrate-utilization and hepatic triglyceride synthesis

4. Determine the effects of whole and fractionated yellow pea flours on the human gastrointestinal microbiome.

CHAPTER II

LITERATURE REVIEW

2.1 THE EFFECTS OF PULSE CROPS ON CIRCULATING LIPID LEVELS

2.1.1 Cholesterol-lowering Effects of Pulse Crops: Total Cholesterol and LDL-Cholesterol

Hypercholesterolemia, specifically elevated LDL-cholesterol (LDL-C) levels, is a risk factor for cardiovascular disease, atherosclerosis and atherogenic dyslipidemia (Braunwald et al., 2001; Cornier et al., 2008; Grundy et al., 2005; Jehle, 2002). Results are mixed regarding the hypocholesterolemic effects of pulse crops. Using doses corresponding to or above the USDA's recommendation (1/2 cup pulses/d), ranging from 104 – 450 g/d cooked/hydrated pulses, chickpeas and beans have induced reductions in total cholesterol (TC) and LDL-C ranging from 4 to 24% and 4 to 19%, respectively (Anderson et al., 1990; Anderson et al., 1984; Finley et al., 2007; Pittaway et al., 2006; Pittaway et al., 2007; Pittaway et al., 2008; Winham et al., 2007). Furthermore, while controlling for diet and energy intake, Frühbeck et al. (1997) observed a 16% reduction in TC and LDL-C in hypercholesterolemic subjects consuming 90 g/d field bean flour for 30 d. Nonetheless, as outlined above, the cholesterol-lowering effects of pulses are controversial. Jenkins et al. (1983b) demonstrated that 140 g/d baked beans had no effect on LDL-C despite a 7% reduction in HDL-C. Similarly, the addition of 130 g/d dry green lentils to controlled Western diets that were formulated to prevent weight change, also failed to effect circulating TC and LDL-C concentrations (Stephen et al., 1995). Finally, neither 110 g/d extruded bean flour and 33 g/d pea fibre had any effect on circulating cholesterol levels, respectively (Oosthuizen et al., 2000; Sandstrom et al., 1994). Altogether, the effects of pulse crops on circulating cholesterol levels are mixed.

Divisive results regarding the hypocholesterolemic effects of pulse crops is testament to the fact that, like fruits and vegetables, individual pulse crops must be evaluated to determine their specific health benefits. Although, lentils, peas, chickpeas and dry beans are all classified as pulse crops, a combination of factors such as the amount and/or types of fibres present as well as whether pulses are consumed as single or multiple dosages dispersed throughout the day could contribute to discrepancies in their cholesterol-lowering efficacy.

Studies suggest that variability in soluble fibre content may explain differences in pulses' cholesterol-lowering efficacy since soluble fibre can bind to endogenous and exogenous cholesterol and prevent its absorption in the gastrointestinal tract (Anderson et al., 1991). A comparison between different pulse crops for their effects on cholesterol levels in pigs receiving a hypercholesterolemic diet demonstrated that baked beans, butter beans and peas induced higher excretion of fecal cholesterol compared to lentils (Kingman et al., 1993). Compared to other pulse crops utilized in the Kingman et al. study, lentils had the lowest levels of total and soluble fibre. Similarly, 130 g/d green lentils had no effect on circulating TC and LDL-C concentrations in humans (Stephen et al., 1995). Martins et al. (2004) revealed that pigs fed raw peas had reduced circulating TC and LDL-C concentrations as well as hepatic TC levels. Pea-fed pigs from the Martins et al. study also demonstrated increased fecal bile output, biliary TC, and bile acid concentrations as well as hepatic 3-hydroxy-3-methylgutaryl-CoA (HMG-CoA) reductase activity. Sequestering bile-acids in the gastrointestinal tract likely induced a cholesterol-lowering effect since bound bile-acids would be excreted rather than re-absorbed. Subsequently, the liver is stimulated to increase expression of LDL-C receptors as a means of enhancing cellular cholesterol levels for use as substrate for bile acid synthesis (Hofmann & Hagey, 2008). The bile-acid sequestering action of pulse crops as a cholesterol-lowering mechanism of action is further supported by studies using non-pulse-derived soluble fibres (Dubois et al., 1995; Jonnalagadda et al., 1993; Weickert & Pfeiffer, 2008). Nonetheless, *in vitro* experiments that simulate the small intestine demonstrated that, compared to baked beans and butter beans, lentils are more effective at binding bile acids

(Elhardallou, 1992) and suggest that other mechanisms work in concert with pulses' lipid-sequestering abilities to facilitate reductions in TC and LDL-C concentrations.

By-products of microbial fermentation of fibres, such as short chain fatty acids (SCFA), could also contribute to pulses' cholesterol-lowering efficacy. SCFA can modulate the expression and/or activity of hepatic genes responsible for cholesterol synthesis. There is evidence that colonic fermentation of certain fibres increases propanoate production which can inhibit HMG-CoA reductase activity (Chen et al., 1984; Levrat et al., 1994). Recently, Finley et al. (2007) demonstrated that, in addition to reduced TC and LDL-C, pinto beans increased the production of isovaleric and isobutyric acids by over 50%, alongside a 50% lower abundance of *Eubacterium limosum*. Derivatives of isobutyric acid have been shown to have hypocholesterolemic effects (Idrees et al., 2009; Morishita et al., 1988). Given that different fibre fractions elicit variable effects on microbial populations as well as proportions of SCFA, further research regarding the prebiotic effects of pulse crops on lipid metabolism is warranted.

Insoluble fibres found in pulse crops may also affect circulating TC and LDL-C levels. Insoluble fibres have been shown to induce satiety which in turn reduces dietary cholesterol intake. Compared to controls, the addition of insoluble fibres, chitosan and cellulose, to high fat/high cholesterol diets in mice prevented a rise in circulating cholesterol concentrations (van Bennekum et al., 2005). In the same study, mice consuming insoluble fibres had decreased exogenous cholesterol intake with no perturbations in cholesterol absorption or excretion (van Bennekum et al., 2005). Given that most of the fibre found in pulse crops is insoluble, insoluble fibre's effect on food intake cannot be excluded as a possible mechanism of action in studies investigating the modulation of circulating TC and LDL-C with pulse crop consumption.

A timing effect has also been demonstrated when pulse crops are utilized to reduce circulating cholesterol levels. Using hyperlipidemic humans, Anderson et al. (1990) administered a single dose of 120 g/d canned beans at noon or divided doses of 120 and 162 g/d canned beans split between lunch and dinner. Results demonstrated that, compared single servings (-8.7%), separated doses of 120 and 162 g/d canned beans produced greater reductions in TC at 11.6 and 10.3%, respectively. Results suggest that the incorporation of pulse crops into multiple meals increases the likelihood of reducing circulating cholesterol levels.

2.1.2 Triglyceride-lowering Effects of Pulse Crops

Hypertriglyceridemia is an independent risk factor for cardiovascular disease (Ferns et al., 2008) and a primary component of atherogenic dyslipidemia, a major morbidity for metabolic syndrome (Cornier et al., 2008). Similar to their effects on TC and LDL-C levels, the triglyceride (TG)-lowering efficacy of pulse crops are mixed. Despite the observation that beans and peas demonstrate TG-lowering efficacy from 11 to 25% (Anderson et al., 1990; Jenkins et al., 1983b; Sandstrom et al., 1994), results are challenged by studies showing negligible efficacy of baked beans, pinto beans and chickpeas on circulating TG levels (Finley et al., 2007; Oosthuizen et al., 2000; Pittaway et al., 2007; Shutler et al., 1989; Winham et al., 2007).

As demonstrated with TC and LDL-C, multiple mechanisms of action regarding pulse crops' effects on TG concentrations have been proposed. Dietary lipid displacement by pulse-derived fibres could reduce TG absorption (Sandstrom et al., 1994). In addition, low post-prandial glycemic responses of pulse crops have been hypothesized to reduce hepatic TG synthesis secondary to a decreased rate of dietary glucose absorption and subsequent insulin responses (Jenkins et al., 1980; Jenkins et al., 1983b; Sandstrom et al., 1994). Oosthuizen et al. (2000) suggested that post-harvest processing such as extrusion could modulate the molecular structures of fibres and disable their lipid displacement

properties. Furthermore, extrusion and canning processes could hydrolyze starch molecules, enhance glucose absorption and cause a subsequent rise in circulating TG levels (Traianedes & O'Dea, 1986). The effects of pulse crops on satiety, food intake and weight loss may also promote healthier circulating TG concentrations. Anderson et al, (1990) observed significant weight loss in volunteers consuming 120 or 162 g/d beans, which was correlated to reductions in TG levels. The relationship between excess body weight and high circulating and hepatic TG concentrations has been demonstrated in human clinical trials (Aguilera et al., 2008; Ferns et al., 2008; Thomas et al., 2005; Viroonudomphol et al., 2003). Propanoate formation from the fermentation of pulse-derived fibres may also modulate hepatic TG synthesis via the aforementioned mechanisms of action discussed with respect to TC and LDL-C. Finally, certain fibres have been shown to modulate levels of glucose-dependent insulinotropic polypeptide (GIP) (Kok et al., 1998) which can increase the expression of adipose lipoprotein lipase (Knapper et al., 1995), a key enzyme responsible for removing TG from the circulation. In addition, rats fed oligofructoses demonstrated a decrease in hepatic VLDL synthesis, the major source of fasted TG (Kok et al., 1996). Overall, the literature attributes the hypotriglyceridemic effects of pulse crops to the ability of pulse-derived fibres to modulate various facets of TG metabolism.

2.2 THE EFFECTS OF PULSE CROPS ON POST-PRANDIAL GLYCEMIA

2.2.1 Benefits of Low Glycemic Foods

Foods that elicit a low post-prandial glycemic response have been identified as protective against cardiovascular disease and type II diabetes via reduced circulating lipid levels, inflammation and the prevention of excessive insulin production and subsequent pancreatic β-islet cell burnout (Esfahani et al., 2009; Jenkins et al., 1995). In addition, low-glycemic diets have demonstrated an ability to induce satiety causing dietary energy restriction and subsequent weight loss by reducing post-prandial ghrelin and insulin while

increasing cholecystokinin levels (Beck et al., 2009; Delargy et al., 1995; Ebbeling et al., 2007; Ebbeling et al., 2003; Juvonen et al., 2009). The fact that pulse crops have consistently demonstrated low post-prandial glycemic responses supports their use as health promoting foods.

2.2.2 Pulse Crops as Low Glycemic Foods

The first post-prandial glycemia studies in the early 1980s utilized lentils as foods with low glycemic indices. Since then, virtually all pulse crops have been identified as low glycemic foods (Foster-Powell & Miller, 1995). Typically it is the high fibre content of pulse crops that is credited as the key contributor to pulse crops' low glycemic index. Viscous fibres interfere with exposure of starch granules to digestive enzymes in the lumen (Jenkins et al., 2000a). However, pulses are also high in protein and anti-nutritional factors, both of which have demonstrated an ability to reduce glycemic response. Proteins are thought to interact with starch granules and prevent their hydrolysis (Jenkins et al., 1987). Similarly, protease inhibitors, amylase inhibitors, phenolic compounds, and phytates protect starch granules and associated proteins from digestion (Thompson et al., 1984). Altogether, low glycemic properties of pulses stem from a combination of factors including fibre, protein and anti-nutritional bioactives.

2.2.3 Effect of Food Processing on Post-Prandial Glycemia

As was demonstrated with lipid-lowering efficacy, stressors associated with cooking and/or food processing procedures may eliminate the low glycemic properties of pulse crops (Tovar et al., 1992). Tovar et al (1992) demonstrated that red kidney beans that were boiled then freeze dried and milled into flour produced a higher post-prandial glucose peak compared to seeds that were boiled or autoclaved. Type II diabetic patients showed significantly higher post-post prandial glycemic responses when fed beans with ruptured cells compared to undamaged cells (Golay et al., 1986). Disruption of cell

structure is a key determinant of starch digestibility (Wursch et al., 1986). The conversion of pulses into flours increases particle surface area exposure to heat and water during the cooking process. The result is increased gelatinization and hydrolyzation of starch granules and greater absorption of glucose from the small intestine (Holm et al., 1989; Holm et al., 1988). Subjects consuming breakfasts containing raw and extruded chickpea flour demonstrated no difference in glycemic response compared to white bread (Johnson et al., 2005). Thus, although pulses are characterized as low glycemic foods when cooked in a traditional manner i.e. boiling, the advent of novel pulse-derived flours as functional ingredients may not confer low-post prandial glycemia. The glycemic index of pulse-derived ingredients, including novel flours, should to be assessed before they are characterized as low glycemic foods.

2.3 THE EFFECTS OF PULSE CROPS ON INSULIN RESISTANCE

2.3.1 Insulin Resistance

Insulin resistance (decreased insulin sensitivity) is characterized as the inability of organs and tissues to respond to normal levels of insulin to maintain healthy circulating glucose concentrations (Savage et al., 2007). Insulin resistance can ultimately lead to type II diabetes (Stumvoll et al., 2005). Encompassing multiple etiologies, insulin resistance has been linked to visceral obesity resulting in higher circulating non-esterified fatty acids, which subsequently interfere with a cascade of cellular processes that facilitate insulin responsiveness and insulin-dependent glucose uptake (Stumvoll et al., 2005). Moreover, chronic insulin resistance can eventually cause insulin-producing pancreatic β-islet cells to "burnout" rendering them unable to manufacture and secrete insulin. In 1992, an editorial by Rupp et al. (1992) emphasized the need for dietary strategies that have insulin sensitizing effects. Since then, chronic ingestion of certain fibres has been shown to reduce insulin resistance.

2.3.2 Effect of Insoluble Fibres on Insulin Resistance

Typically, insoluble fibres demonstrate an insulin sensitizing effect (Weickert & Pfeiffer, 2008) and given that pulse crops are good sources of insoluble fibre, they are ideal candidates for investigating dietary interventions that reduce insulin resistance. Unfortunately studies utilizing pulse crops as insulin sensitizing agents have yet to surface. However, other sources of insoluble fibre have demonstrated significant reductions in insulin resistance. Overweight and obese subjects consuming 31.2 g/d cereal-derived insoluble fibre over 3d demonstrated an 8% improvement in insulin sensitivity (Weickert et al., 2006). Compared to placebo, Robertson et al. (2005) observed a 14% increase in insulin sensitivity following four wks of 30g/d resistant starch supplementation. In addition, the same study revealed that post-prandial skeletal muscle-mediated glucose uptake was significantly increased following a liquid meal tolerance test (Robertson et al., 2005). Other studies using a second meal design demonstrated that insoluble fibre consumption during an initial meal elicited reduced insulin secretion during a subsequent meal consumed at a later time; thus suggesting that insoluble fibres have insulin sensitizing properties (Robertson et al., 2003; Weickert et al., 2005). Furthermore, prospective cohort studies and meta-analyses suggested that, rather than soluble fibre, insoluble fibre reduce the risk of type II diabetes (de Munter et al., 2007; Schulze et al., 2007). Given that pulse crops are high in insoluble fibre, studies that evaluate the effects of pulse crops as well as their functional ingredient-derivatives on insulin resistance are warranted.

2.4 PULSE CROPS AND BODY COMPOSITION

Studies demonstrate that high fibre diets are associated with lower BMIs and lower body fat content compared to men and women consuming low fibre diets (Appleby et al., 1998; Miller et al., 1994). Mice fed energy restricted diets alongside mung beans as a source of carbohydrate had a ≈60% reduction in visceral fat mass compared to mice fed instant

oatmeal with sugar, respectively (Morris & Zemel, 2005). Researchers hypothesize that reduced food intake alongside lower post-prandial hyperglycemia and insulin responses decreased visceral lipid deposition (Morris & Zemel, 2005). Long-term consumption of high fat diets with added chickpeas prevented excessive visceral adipose tissue deposition and increased leptin mRNA expression as well as activities of muscle and hepatic lipoprotein lipase compared to similar diets without chickpeas (Yang et al., 2007). Moreover, compared to soluble fibre, mice fed Western diets fortified with insoluble fibre noted decreased weight gain and fat mass despite no differences in energy intake between groups (Isken et al., 2010). Recently, Abete et al. (2009) demonstrated that legumes increase mitochondrial oxidation in humans, suggesting a shift in macronutrient oxidation. Depending on how carbohydrates, fat and protein are utilized to sustain energy requirements, lipid and carbohydrate storage could be increased or decreased. However, given that subjects in the Abete et al. (2009) study were fed hypocaloric diets, it cannot be determined how much of the effect was secondary to legume consumption or energy restriction.

Given that visceral adiposity is a risk factor of hyperlipidemia, insulin resistance and type II diabetes (Brochu et al., 2008; Despres, 1993; Goodpaster et al., 1997; Slyper, 1998), bioactives that modulate regional adipose deposition are of interest. Stimulation of certain estrogen receptors, in part, determines how lipid is stored. For example, studies demonstrate that stimulation of estrogen alpha receptors (ERα) reduce lipolysis in subcutaneous adipose which promotes subcutaneous fat deposition (Pedersen et al., 2004; Richelsen, 1986). Conversely, estrogen beta receptors (ERβ) increase subcutaneous adipose lipolysis which impedes fat deposition (Pedersen et al., 2004; Richelsen, 1986). Given that the soy-derived phytoestrogens preferentially bind ERβ (Morito et al., 2001), Sites et al. (2007) suggest that the observed reduction in subcutaneous fat at -14.73± 22.26 cm^2 compared to placebo (+22.91 ± 28.58 cm^2) in postmenopausal women consuming soy-derived phytoestrogens was secondary to stimulation of ERβ. The physiological effects of pulse-derived phytoestrogens have yet to be determined. Nonetheless, relative to soy beans, pulse crops such as kidney and alfalfa beans contain

considerably lower ratios of genestein-to-secoisolariciresinol (Mazur, 1998). Secoislariciresinol is metabolized and absorbed as enterolactone, which preferentially stimulates ERα (Penttinen et al., 2007), and could favor subcutaneous adipose deposition over visceral adipose deposition. Facilitating subcutaneous adipose production would propagate a healthier regional adipose tissue profile with lower prevalence of risk factors that are associated with excess ectopic fat. Overall, evidence suggests that chronic consumption of pulse crops could modulate adipose tissue distribution.

2.5 PULSE CROPS AND TRIGLYCERIDE SYNTHESIS

Effects of pulse crops on TG synthesis are unknown. Given that PPAR-α expression decreases TG synthesis (Konig et al., 2009), and Isken et al. (2010) have demonstrated that insoluble fibres enhance PPAR-α expression, it can be hypothesized that insoluble fibre-rich pulse crops can modulate de novo lipogenesis. In addition, diets containing high levels of readily digestible carbohydrate are known to increase TG synthesis, causing post-prandial hyperlipidemia (Hudgins et al., 2000; Parks et al., 2008). Thus, given that pulses are high in insoluble fibre, while possessing a low glycemic index, it can be hypothesized that acute or chronic pulse consumption can reduce post-prandial TG synthesis. Nonetheless, the effect of pulse-derived flours on TG synthesis must be evaluated given their susceptibility to biochemical modification during processing and cooking.

2.6 PULSE CROPS AND POST-PRANDIAL ENERGY EXPENDITURE AND SUBSTRATE UTILIZATION

Very few studies have examined the effects of pulse crops on post-prandial energy expenditure and substrate utilization. However, given that protein produces the highest thermic effect of food (Robinson et al., 1990) and the fact that pulse crops are good sources of protein, enhanced thermogenesis with pulse consumption is plausible. The aforementioned study described above by Abete et al. (2009) demonstrated enhanced mitochondrial oxidation in subjects consuming a legume-rich diet. Conversely, diets fortified with fibre have been shown to reduce post-prandial heat production, most likely secondary to blunted carbohydrate absorption (Khossousi et al., 2008). The effects of whole and fractionated pulse crops on post-prandial energy expenditure warrant further exploration. By delineating their effects on thermogenesis, different pulse fractions could become highly specialized food ingredients in specific patient populations.

Similar to energy expenditure, effects of pulse crops on substrate utilization remain unknown. As described above, given their high protein content, protein oxidation would be expected to increase with the consumption of protein-rich fractions of pulse crops. In the study described above by Isken et al.,(2010) whereby insoluble fibre decreased excessive weight gain and fat accumulation in mice, insoluble fibre also increased the expression of transcription factors that foster TG β-oxidation including hepatic forkhead transcription factor (Foxa2), peroxisome proliferator-activated receptor (PPAR)-γ coactivator β (Pgc-1β), and PPAR-α (Isken et al., 2010). Mice fed insoluble fibre also demonstrated reduced TG accumulation in liver homogenates compared to mice fed soluble fibre (Isken et al., 2010). Conversely, pulse crop fractions comprised primarily of fibre would be expected to reduce carbohydrate oxidation via decreased carbohydrate absorption and facilitate a higher reliance on fat oxidation during the post-prandial period. Again, further examination into the effects of whole and fractionated pulse crops on post-prandial substrate utilization is needed.

2.7 THE PREBIOTIC EFFECTS OF PULSE CROPS

2.7.1 Importance of a Healthy Gastrointestinal Microbiome

As of late, the impact of the intestinal microbiome on local colonic as well as systemic health has received substantial attention from the scientific community. Studies show that a diverse milieu of gastrointestinal microflora propagates a healthy phenotype. For example, a metagenomic comparison of colonic microflora demonstrated that the proportion bacterial DNA belonging to the phylum Firmicutes was 22% in patients with Crohn's disease compared to 49% in healthy volunteers (Manichanh et al., 2006). Conversely, the number operational taxonomic units from Proteobacteria, Bacteroidetes and Actinobacteria did not differ between groups (Manichanh et al., 2006), suggesting that Firmicutes help maintain intestinal health. Gut microbes have also been shown to modulate immune function, obesity, and inflammation (Backhed et al., 2004; Kadooka et al., 2010; Laitinen et al., 2009; Ley et al., 2005; Lomax & Calder, 2009a). Mice exposed to antibiotics produced a substantial reduction in gastrointestinal microflora alongside a down regulation of toll-like receptors 2 and 4 in peritoneal macrophages, impairing their immune-response to lipopolysaccharide (Umenai et al., 2010). In addition, lean germ-free mice transplanted with unfractionated caecum microbes from conventionally raised mice gained substantial weight, while germ-free mice not inoculated remained lean (Backhed et al., 2004). Moreover, inoculated mice demonstrated less food intake and increased energy expenditure compared to uninoculated germ-free mice (Backhed et al., 2004). Similarly, germ-free mice consuming a Western high-fat diet remained lean until inoculated with gut microflora from obese mice (Backhed et al., 2007). Finally, mice consuming high-fat diets demonstrated changes in gastrointestinal microbial populations and developed metabolic endotoxemia via increased gut permeability and high levels of circulating endotoxin (Cani et al., 2008). However, when mice on the same high-fat diet were subjected to antibiotic treatment, which drastically reduced caecal microbial populations, endotoxemia did not ensue (Cani et al., 2008). Researchers hypothesized that high fat diets elicit unfavorable changes in microbial populations which increase gut

permeability to inflammatory reagents such as lipopolysaccharide (Cani et al., 2008). Studies demonstrate that gut microflora promote colonic as well as systemic health.

2.7.2 Pulse Crops as a Prebiotics

Typically, probiotic supplementation is utilized as a means to modulate gastrointestinal microbial populations to improve the health of the host. Probiotic supplementation have been shown to induce remission in patients with ulcerative colitis (Bibiloni et al., 2005), improve systemic immune responses to vaccinations, incidence of infection (Boge et al., 2009; Lin et al., 2009; Soh et al., 2010), and reduce metabolic endotoxemia associated with high-fat diets (Cani et al., 2007). Recent studies suggest that probiotics can reduce visceral obesity and improve glucose tolerance in humans (Kadooka et al., 2010; Laitinen et al., 2009). Nonetheless, another way to modulate the gastrointestinal microbiome is through the use of prebiotics.

Unlike probiotics, prebiotics *"are nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health"* (Gibson & Roberfroid, 1995). Most prebiotics consist of fermentable fibres. In addition to the benefits of increasing levels of "good" bacteria, metabolites of fibre fermentation, such as SCFA, are believed to provide systemic health benefits as outlined above. Nonetheless, compared to probiotics, few studies have investigated the effects prebiotics on gastrointestinal microflora as well as their health benefits. Recently, Nishimura et al. (2010) demonstrated that a prebiotic supplement derived from cellulose reduced histological evidence of dextrane-sulfate-induced colitis in mice alongside a reduction in the proinflammatory cytokines: tumor necrosis factor alpha (TNF-α), IL (interleukin)-1β, IL-17, and interferon inducible protein -10. Pigs administered a soluble fibre supplement derived from the chemical manipulation of maize and wheat starches significantly increased levels of Bifidobacterium spp., Peptostreptococcus spp. and

Fusobacterium spp. compared to pigs supplemented with dextrose (Pouillart et al., 2010). Moreover, after induction of colitis with trinitrobenzene sulfonic acid, pigs consuming the prebiotic experienced less weight loss, fecal occult blood and reduced histological evidence of cell damage (Pouillart et al., 2010).

Despite their high fibre content, few studies have examined the prebiotic potential of pulse crops. One study by Finley et al, (2007) demonstrated only moderate perturbations in fecal microbial populations and SCFA production after subjects consumed ½ cups/d (130 g/d) mung beans. However, subjects were permitted to consume their habitual diets in addition to the mung bean treatments. As outlined above, diet composition can profoundly modulate the gastrointestinal microbiome and could counteract the effects of prebiotic treatments. Thus, random clinical trials that impose control over the underlying diet would better delineate the prebiotic effects of prebiotic treatments. Finally, given that analysis of the gut microbiome demonstrated the presence of amino acid digesting bacteria (Macfarlane et al., 1992), it cannot be discounted that the protein fraction of pulse crops could elicit a prebiotic effect. Moreover, gluten free diets have been shown to modulate gastrointestinal microbial populations (De Palma et al., 2009). Accordingly, further studies investigating the prebiotic effects of whole and fractionated pulse crops are warranted.

2.8 CONCLUSION

The nutrient profile of pulse crops, high in insoluble and soluble fibres, protein and low in fat, suggests that these food materials would elicit beneficial effects on circulating lipid levels, post-prandial glucose tolerance, TG synthesis, body composition, post-prandial energy expenditure and substrate utilization, as well as induce favorable effects on the gastrointestinal microbiome. Nonetheless, results regarding the direct effect of pulse crops on the above endpoints are mixed. Even with clinical studies investigating reductions in circulating LDL-C and TG concentrations, where the largest body of pulse crop-based intervention studies exists, there is substantial variability in results. One can only speculate as to why pulses elicit inconsistent results on health outcomes. However, implementation of highly controlled interventions where background diet composition and energy intake are fixed may help delineate the true effects of pulse crops on indices of cardiovascular disease, diabetes and obesity as well as the gastrointestinal microbiome. Finally, such trials are especially needed to assess the efficacy of novel pulse-derived food ingredients, such as flours, which represent an innovative means for incorporating pulse crops into diets.

CHAPTER III

WHOLE AND FRACTIONATED YELLOW PEA FLOURS REDUCE FASTING INSULIN AND INSULIN RESISTANCE IN HYPERCHOLESTEROLEMIC AND OVERWEIGHT HUMANS

Christopher P.F. Marinangeli[1] and Peter J.H Jones[1]

[1]The Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, 196 Innovation, Winnipeg, Manitoba, R3T 2N2

To whom correspondence reprint requests should be directed:

Dr. Peter J.H. Jones, Ph.D.

Richardson Centre for Functional Foods and Nutraceuticals

University of Manitoba

196 Innovation Drive

Winnipeg, MB R3T 2N2, Peter_jones@umanitoba.ca

Tel: 1+204 474 8883

Fax: 1+204 474 7552

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3.1 ABSTRACT

The objective was to compare whole yellow pea flour (WPF) and fractionated yellow pea flour (FPF) (hulls only) for their ability to reduce risk factors associated with CVD and diabetes in overweight hypercholesterolemic individuals. Using a crossover design, 23 hypercholesterolemic overweight men and women received two treatment muffins/day containing WPF, FPF, or white wheat flour (WF) for 28 day, followed by 28 day washout periods. Daily doses of WPF and FPF complied with the USDA's recommended level of intake of ½ cups pulses/day (≈ 50 g/day). Dietary energy requirements were calculated for each study subject and volunteers were only permitted to eat food supplied by study personnel. Fasting insulin, body composition, urinary enterolactone levels, post-prandial glucose response, as well as fasting lipid and glucose concentrations, were assessed at beginning and end of each treatment. Insulin concentrations for WPF (37.8±3.4 pmol/L, $p=0.021$) and FPF (40.5±3.4 pmol/L, $p=0.037$) were lower compared to WF (50.7±3.4 pmol/L). Insulin homeostasis model-assessment showed that consumption of WPF and FPF decreased ($p<0.05$) estimates of insulin resistance compared to WF. Android-to-gynoid fat ratios in participants in women were lower ($p=0.027$) in the WPF (1.01±0.01) group compared to the WF group (1.06±0.01). Urinary enterolactone levels tended to be higher ($p=0.087$) with WPF compared to WF. Neither treatment altered circulating fasting lipids or glucose concentrations. In conclusion, under a controlled diet paradigm, daily consumption of whole and fractionated yellow pea flours at doses equivalent to ½ cups yellow peas/day reduced insulin resistance, while WPF reduced android adiposity on women.

3.2 INTRODUCTION

Pulses are annual leguminous crops that include lentils, beans, chickpeas and yellow peas. Pulse crops are high in protein, antioxidants, and fibre and low in fat and calories, all factors that contribute to reduction of cardiovascular disease (CVD) and type 2 diabetes mellitus risk (Hu & Willett, 2002; Willett, 2008). The USDA recommends that adults consume ½ cup of legumes/day, including pulses, as part of a healthy diet. Typically, pulse consumption is recommended to diabetics as a means of controlling post-prandial glycemia. However, animal and human studies have noted that consumption of chickpeas, beans and lentils also reduce circulating lipid and glucose concentrations, while improving body composition by decreasing percent fat mass (Anderson & Major, 2002; Leterme, 2002; Yang et al., 2007). Although satiety and reduced food intake are believed to contribute to health benefits associated with pulse crops (Venn & Mann, 2004), the effects of individual pulse crops remain poorly characterized.

Health benefits of yellow peas have been given little attention in human clinical trials, despite being a low-cost commodity with widespread consumption around the world. Yellow peas consist of an outer layer known as the seed coat or hull and an inner portion termed the cotyledon. The seed coat is 85 to 90% fibre, while the cotyledon is higher in protein and unsaturated fat. In addition, yellow peas are a source of secoisolariciresinol (Mazur, 1998), a phytoestrogen that is metabolized by colonic microflora to enterolactone (Valentin-Blasini et al., 2005). Investigating how sub-components of yellow peas impact on indices of health is likely to shed light on which portion of yellow peas possesses the most health benefits, bringing awareness to yellow peas as a dietary approach for CVD and diabetes prevention.

Evaluating and classifying how various fractions of pulse crops impact on health will serve as a rational for isolating and concentrating their components for inclusion into diets that do not utilize pulse crops as staple foods. The production of novel pulse-derived flours serves as a new platform for administering pulse fractions enabling new and flexible approaches for incorporating pulse-derived components into North American diets. As such, the objective of this study was to evaluate the efficacy of whole and fractionated yellow pea flours in reducing risk factors associated with CVD and diabetes while controlling food and energy intake.

3.3 MATERIALS AND METHODS

3.3.1 Subjects

Twenty-nine hypercholesterolemic, overweight (BMI 25 to 40 kg/m^2) men and women were recruited from the Winnipeg area using newspaper and radio advertisements. Baseline characteristics of subjects are summarized in **Table 1.** Exclusion criteria included smoking, the use of prescription and natural lipid lowering therapies, history of myocardial infarction, coronary artery bypass, angina, congestive heart failure, inflammatory bowel disease, pancreatitis, renal disease, diabetes and a history of chronic alcohol usage (> 2 drinks/day). Individuals with cancer, eating disorders and those who reported expending > 16 747 kJ/week through exercise were also excluded from participation. Participants taking medications for other ailments were included providing their daily dose of medication remained stable for the duration of the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the University of Manitoba's Biomedical Research Ethics Board (B2006: 129). Written informed consent was obtained from all subjects. This study was registered at www.clincialtrials.gov, ID NCT00839774 (February 6, 2009).

3.3.2 Treatments

Treatments consisted of whole pea flour (WPF), fractionated pea flour (hulls only) (FPF) and white wheat flour (control) (WF). Treatment flours were administered to subjects in banana and apple muffins. Muffin recipes were developed at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) clinical kitchen. WPF and FPF flours were obtained from Best Cooking Pulses Inc. (Portage la Prairie, MB, Canada), while the

Table 1. Baseline characteristics of study subjects

Criteria	Men n = 10	Women n = 19
Age	51.8 ± 12.3	52.3 ± 10.0
Weight (kg)	95.0 ± 18.6	80.4 ± 12.1
BMI (kg/m^2)	31.7 ± 5.3	29.4 ± 3.5
TC (mmol/L)[§]	5.63 ± 0.53	6.26 ± 0.81
LDL-C (mmol/L)[§]	3.32 ± 0.96	3.92 ± 0.75
HDL-C (mmol/L)[§]	1.11 ± 0.14	1.52 ± 0.33
TG (mmol/L)[§]	2.52 ± 1.31	1.85 ± 1.04
Glucose (mmol/L)[§]	5.79 ± 1.32	4.84 ± 0.49

[§] Fasted levels in serum

Abbreviations: TC, total cholesterol, LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides

WF was obtained from a local supermarket. Pea treatment flours were analyzed by Medallion Labs (Minneapolis, MN, USA) to determine their nutrient composition (**Table 2**). The composition of WF was determined by available food analysis tables (Brault Dubuc & Caron-Lahaie, 1994). Current USDA recommendations for pulse consumption are ½ cups/day, which is ≈ 50 g dry whole yellow peas (USDA.gov, 2009). Each WPF treatment muffin was formulated to contain ≈ 25 g, 50% the USDA recommended daily

serving of pulses. Given that two WPF treatments were equivalent to ½ cups/day dry yellow peas (50 g), subjects were required to eat two treatment muffins/day. The FPF treatment muffins were formulated based on total pea-derived fibre content of the WPF muffin since the pea hull flour used in the present study was approximately 84.2 % fibre (**Table 2**). Hence, the amount of fractionated flour added to the FPF muffin recipe was adjusted to equal the amount of fibre in the WPF treatment (**Table 3**). The balance of the muffin weight was white wheat flour. Adjusting FPF treatments to contain the same amount of pea fibre as the WPF treatment enabled distinguishing of whether it was the fibre component of the USDA's recommended intake of pulses that conferred health benefits or the cotyledon component of WPF. The WF muffin was solely comprised of white wheat flour. Xanthin gum was added to all muffin recipes at 2.0 % of total flour weight to compensate for WPF and FPF flours lacking gluten.

3.3.3 Study Design

The present study used a single blind, crossover design. The study consisted of three, four-week study phases, each followed by a four-week washout. To determine the treatment order within each subject, participants were randomly assigned to one of the six possible treatment sequences.

Table 2. Composition of treatment flours and treatment muffins

Treatment Flour	**WPF**[1§]	**FPF**[1§]	**WF**[2]
Energy (kJ)/100 g	1 281	343	1 482
Total carbohydrate (%)	66.3	89.0	79.4
Available carbohydrate (%)	50.7	12.7	76.3
Fibre (%)	17.6	84.2	3.1
Soluble (%)	2.0	7.9	1.8
Insoluble (%)	15.6	76.3	1.3
Total fat (%)	1.84	0.63	1.0
Polyunsaturated fat (%)	0.98	0.31	0.41
Monounsaturated fat (%)	0.44	0.13	0.09
Saturated (%)	0.34	0.16	0.15
Protein (%)	21.6	6.39	10.3
Treatment Muffins	**WPF**[1]	**FPF**[1]	**WF**[2]
Whole pea flour per muffin (g)	26.4	-	-
Fractionated pea flour per muffin (g)	-	6.0	-
White wheat flour per muffin (g)	-	20.4	26.4
Weight per muffin (g)	91	91	91
Energy (kJ)	821.5	816.9	888.5
Carbohydrate (g)	30.4	33.8	33.0
Total fibre (g)	5.8	6.9	2.0
Soluble fibre (g)	1.7	2.0	1.6
Insoluble fibre (g)	4.1	4.8	0.3
Total fat (g)	7.4	7.1	7.1
Polyunsaturated fat (g)	3.8	3.7	3.7
Monounsaturated fat (g)	3.2	3.1	3.1
Saturated fat (g)	0.9	0.8	0.8
Protein (g)	7.1	3.9	4.1

Abbreviations: WPF, whole pea four; FPF, fractionated pea flour; WF, white wheat flour.

[1] Treatment flours analyzed at Medallion Labs (Minneapolis, Minnesota, USA)

[§] Analysis of total, soluble and insoluble fibre was completed by Medallion Labs using AOAC method 991.43.

[¥] Flour composition determined using available nutritional tables (Brault Dubuc and Caron-Lahaie, 1994)

3.3.3.1 Diet and Treatment Protocol

For the duration of each study phase, subjects only consumed food provided by the RCFFN clinical research kitchen. The nutrient composition of the study diet was analyzed using Food Processor version 7.71 (ESHA Research, OR, USA) as is summarized in **Table 3**. The background diet was in accordance with the American Heart Association's NCEP-Step 1 diet. Diets did not contain any pulse products besides the treatment muffins. To prevent bodyweight change for the duration of each study phase, daily energy requirements for each subject were determined by calculating individual resting metabolic rates using the Mifflin Equation (Mifflin et al., 1990), then applying an activity factor of 1.7. The amount of food each participant received was based on individual calculated energy requirements. The average caloric content of two treatment muffins (approximately 1,675 kJ) was subtracted from the total calculated energy requirements to compensate for the energy intake from WPF, FPF and WF. Body weights were assessed daily. If a subject's weight increased or decreased in the first week of each study phase, energy intake was adjusted accordingly. Each morning, study participants were required to visit the RCFFN Clinical Research Unit to consume breakfast and one banana flavored treatment muffin under supervision. Participants would then leave the unit with a cooler containing the remainder of their meals and snacks alongside an apple-flavored treatment muffin to consume with dinner. Returned empty, unwashed food containers were used to monitor compliance.

3.3.3.2 Blood Collection Protocol

Twelve-hour fasting serum and plasma blood samples were collected on days 1, 2, 28 and 29 of each study phase. Blood was centrifuged at 3000 rpm at 4°C to separate serum and plasma from red blood cells, which were then stored at -80°C until analyzed.

Table 3. Energy requirements and background diet composition for study participants

Diet Composition	
Carbohydrate (%)	55%
Protein (%)	15%
Fat (%)	30%
Diet Summary[§]	
Energy (MJ/d)	12.56
Carbohydrate (g)	403.7
Protein (g)	113.2
Fat (g)	97.3
Polyunsaturated fat (g)[¥]	19.6 (6%)
Monounsaturated fat (g)[¥]	20.8 (6%)
Saturated fat (g)[¥]	25.1 (8%)
Dietary fibre (g)	18.8
Soluble fibre (g)	4.8
Cholesterol (mg)	238.8

[§] Nutritional summary is based on a 12.56 MJ/day diet

[¥] Brackets denote percent of total energy

3.3.3.3 Fasting Insulin Values and Insulin Homeostasis Modeling Assessment (HOMA)

Fasting plasma insulin concentrations were determined using ELISA (Millipore, Linco Research, MO, USA). Average CV% between duplicate samples was 1.0%. Resultant insulin values were transformed from μU/mL to pmol/L by multiplying values by a factor of 6.0 (Volund, 1993). Insulin HOMA was utilized as an estimate for %β-cell function and insulin resistance (IR). HOMA values were calculated using methods outlined by Matthews et al. (1985). Baseline and endpoint insulin parameters were reported as the average between day 1 & 2 and day 28 & 29 values, respectively.

3.3.3.4 Body Composition Protocol

Body composition, including percent total fat mass (%TFM), total fat mass (TFM) and total lean mass (TLM), as well as percent android and gynoid fat, were assessed on day 1 and 29 of each study phase using fan beam dual energy X-ray absorptiometry (DXA) (Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA). Body composition data including %TFM, TFM, TLM, android fat and gynoid fat deposition were determined using Encore 2005 software version 9.30.044 (GE Healthcare, Madison, WI, USA). Encore 2005 also calculated android-to-gynoid fat ratios by dividing % android fat by % gynoid fat.

3.3.3.5 Glucose Response Protocol

Glucose responses to the breakfast meal and treatment were determined during the first and last week of each study phase. Fasting glucose concentrations were determined using a finger-prick blood glucose monitor (Accu-chek plus, Roche Diagnostics, Laval, QC, Canada). Subjects had 40 min to eat their energetically-calculated breakfast and one treatment muffin (≈ ¼ cup of peas). Glucose concentrations were assessed prior to and at 40, 70, 130 and 190 min following the initiation of breakfast. At least two blood glucose readings were taken at each time point. A third measurement was taken if glucose values were > 0.3 mmol/L apart. Finally, two quality assurance solutions containing known amounts of either high glucose or low glucose levels were used daily to ensure glucose monitors produced accurate results.

Incremental areas under the curve (IAUC) for glucose response curves were determined using methodologies outlined by Wolever et al. (1986). Briefly, the area below baseline was subtracted from the total IAUC values. If the data points fell below baseline, only areas above the baseline value were considered.

3.3.3.6 Urinary Enterolactone Levels

Morning fasted urine samples were collected on day 29 of each study phase. Subjects were instructed not to void their bladder at home prior to arriving at the RCFFN. Urine samples were aliquoted and stored at -80°C until analysis. Urinary enterlactone levels were analyzed by competitive enzyme immunoassay (EIA) (Cayman Chemical, Ann Arbor MI, USA). All samples were diluted 1000x prior to EIA analysis. Urinary creatinine levels were determined enzymatically (Vitros 350, Ortho-clinical Diagnostics Inc. Rochester NY, USA) and used to normalize urinary enterolactone values.

3.3.3.7 Fasting Serum Lipid and Glucose Concentrations

Day 1, 2, 28 and 29 serum total cholesterol (TC), HDL-cholesterol (HDL-C), triglyceride (TG) and glucose concentrations were analyzed enzymatically (Vitros 350, Ortho-clinical Diagnostics Inc. Rochester NY, USA). LDL-cholesterol (LDL-C) concentrations were calculated using the Friedewald Equation (Friedewald et al., 1972). Baseline and endpoint lipid and glucose parameters were reported as the average between day 1 & 2 and day 28 & 29 values, respectively.

3.3.4 Statistical Analysis

Results are presented as the mean ± SEM. Differences in endpoints values between treatment groups for body composition, TC, LDL-C, TG, HDL-C, glucose, insulin, and HOMA data were analyzed using linear mixed model analysis of variance (ANOVA) with subject as a random factor. Normality was assessed using the Shapiro-Wilk test, as well as evaluating linearity and clustering in normal and disturbed Q-Q plots, respectively. Circulatory TG, HDL-C concentrations, HOMA variables and urinary entrolactone levels did not follow a normal distribution and were log-transformed for statistical analysis. For reporting purposes, means and SEMs were back-transformed to physiological values. For glucose response, total effect of treatment over time was assessed using repeated measures. For all ANOVA, estimated means were compared using the Bonferroni post hoc test when significant differences were noted. Intra-treatment differences between start-phase (average between day 1 & 2) values and end-phase (average between day 28 & 29) values were determined using paired t tests. A level of significance at $p<0.05$ was used in all analyses. Data were analyzed using SPSS software version 11.5 (SPSS Inc., Chicago, IL, USA).

3.4 RESULTS

3.4.1 Subject Dropout and Compliance

Of the 29 individuals who participated in the study, 23 completed the entire 12 week trial. Two subjects discontinued the study for medical reasons, while four subjects dropped out for personal reasons. After dropouts, the number of men and women in the study were 7 and 16, respectively. One subject was taking aspirin for the duration of the study. No other subject was consuming over-the-counter or prescription medications over the course of the study. Statistical analysis for all measurements revealed no effect of gender, age or an age by gender interaction.

3.4.2 Fasting Insulin and Insulin Homeostasis Modeling Assessment for Insulin Resistance and %β-cell Function

End-phase fasting insulin concentrations were reduced 25.4% and 20.1% during WPF (37.8±3.4 pmol/L, $p=0.021$) and FPF (40.5±3.4 pmol/L, $p=0.037$) treatments, respectively, compared to WF (50.7±3.4 pmol/L) (**Figure 1A**). No differences were observed for endpoint insulin concentrations between WPF and FPF treatments. Furthermore, within-phase analysis revealed that WPF and FPF significantly reduced fasting insulin by 13.5±4.8% ($p=0.009$) and 9.8±4.8% ($p=0.045$) from baseline compared to WF (+6.8%) (**Figure 1B**). No differences in percent change between baseline and endpoint were observed between WPF and FPF.

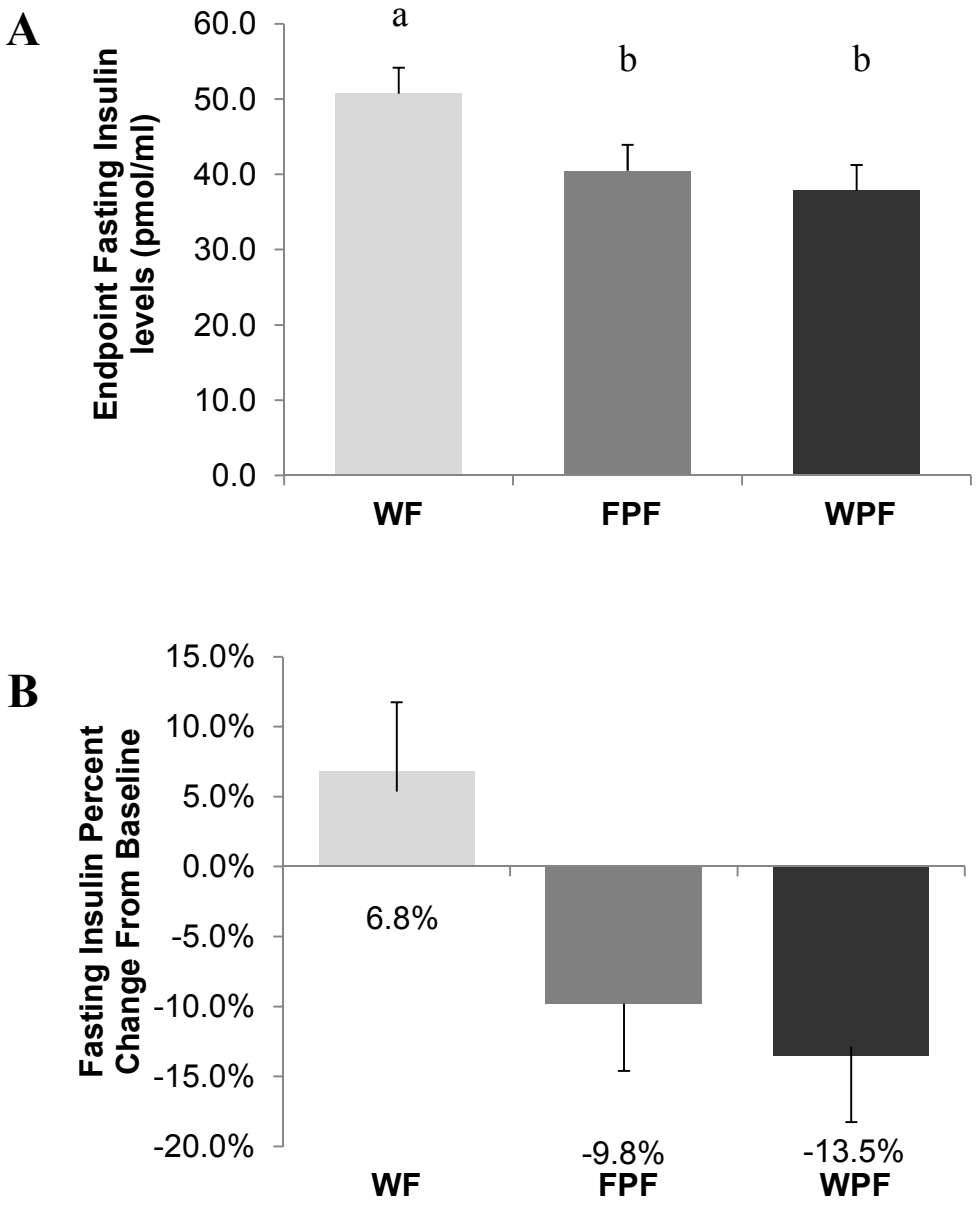

Figure 1 A-B. The effect of treatments on fasting insulin concentrations. Abbreviations for treatments: WF, white wheat flour; FPF, fractionated pea flour; WPF, whole pea flour. Endpoint values represents data after 29 days of treatment while percent change from baseline represents the difference between Day 1/2 and day 28/29 values. Endpoints with different letter subscripts are significantly different from each other. Statistical significance was set at $p<0.05$ (n=23).

Homeostasis modeling assessment for insulin revealed that estimates of IR were reduced 25% in the WFP (1.5±0.2, *p=0.012*) and FPF (1.5±0.2, *p=0.036*) groups, respectively, compared to the WF (1.9±0.2) group (**Figure 2**). However, estimates of %β-cell function were not different between groups at 83.4±8.4%, 91.8±8.4%, and 105.4±8.4% for WPF, FPF and WF, respectively. Percent differences from baseline were not significantly different between WPF, FPF and WF treatments for insulin HOMA for or HOMA-based markers of %β-cell function and IR, respectively.

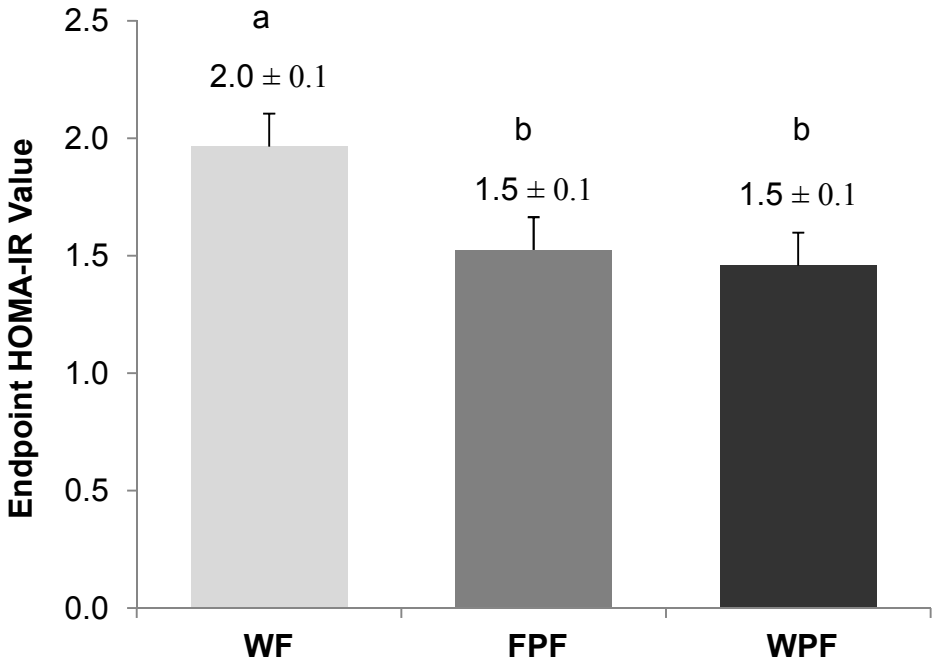

Figure 2. Endpoint homeostasis modeling assessment (HOMA) for insulin resistance after consuming WF, FPF and WPF treatments. Abbreviations for treatments: WF, white wheat flour; FPF, fractionated pea flour; WPF, whole pea flour. Endpoint values represent data after 28 days of treatment. Endpoints with different letter subscripts are significantly different from each other. Statistical significance was set at $p<0.05$(n=23).

3.4.3 Body Composition

Intra-phase body weights between day 1 and day 29, calculated by averaging daily body weights from each day of active phases, were stable at 84.9±0.6, 85.7±0.6 and 84.3±0.6 kg for WPF, FPF and WF, respectively. DXA analysis revealed that WPF, FPF and WF had no effect on endpoint values or calculated intra-treatment percent differences for %TFM, android fat or gynoid fat. While no changes were noted between WF (1.14±0.01) and WPF (1.10±0.01) compared to FPF (1.11±0.01), there was a trend toward a reduction (*p=0.059)* in the android-to-gynoid ratio for WPF versus WF. However, when stratified by gender, females (n=16) administered WPF (1.01±0.01) demonstrated a 4.7% reduction (*p=0.027*) in android-to-gynoid ratios compared to when they were receiving WF (1.06±0.01) (**Figure 3**).

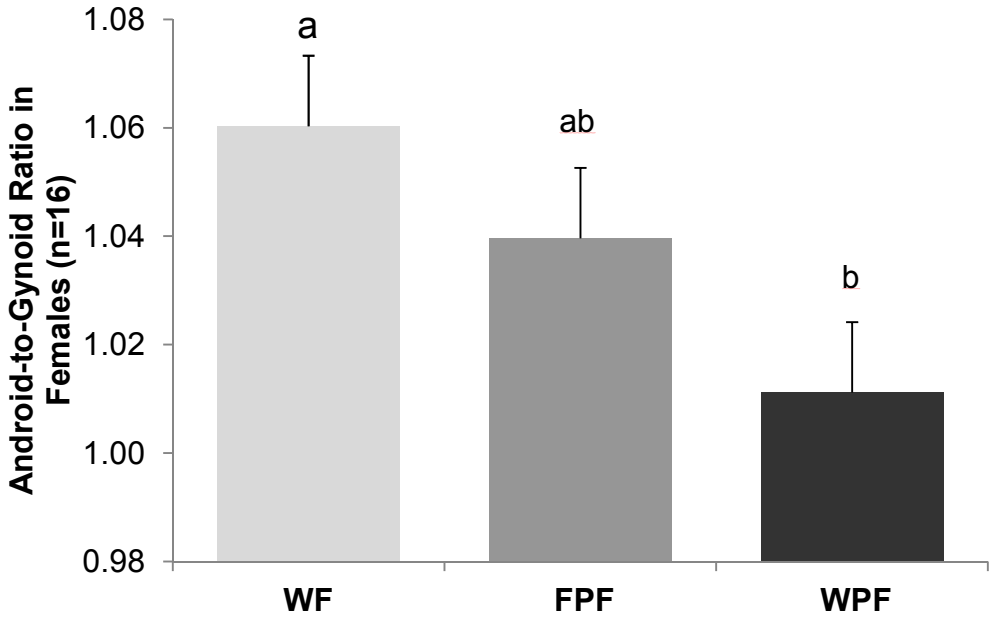

Figure 3. Android-to-gynoid ratio in women (n=16) after 28 days of treatment. Abbreviations for treatments: WF, white wheat flour; FPF, fractionated pea flour; WPF, whole pea flour. Endpoints with different letter subscripts are significantly different from each other. Statistical significance was set at $p<0.05$.

3.4.4 Post-prandial Glucose Response

No treatment differences were observed in postprandial glucose IAUC for endpoint and percent change from baseline.

3.4.5 Urinary Enterolactone Levels

Urinary enterolactone levels tended to be higher (*p=0.084*) in the WPF (12.0±2.7 nmol/mmol creatinine) group compared to the WF group (10.4±2.7 nmol/mmol creatinine). Levels of enterolactone in the FPF group was 11.8±2.7 nmol/mmol creatinine.

3.4.6 Fasting Serum Lipid and Glucose Concentrations

Within-group reductions in TC and LDL-C were observed in all three treatment groups, alongside decreases in HDL-C in the WPF and WF groups compared to baseline (**Table 4**). Fasting glucose decreased in the FPF group compared to baseline. However, no effect on fasting serum TC, LDL-C, HDL-C, TG or glucose concentrations were observed between treatment groups (**Table 4**).

Table 4. Summary of fasting serum lipid and glucose values

Endpoint	WPF	FPF	WF	P value[§]
TC				
Start phase (mmol/L)	6.16 ± 0.16	6.12 ± 0.15	6.14 ± 0.17	
End phase (mmol/L)	5.50 ± 0.13[c]	5.51 ± 0.13[b]	5.42 ± 0.13[c]	0.678
Change (%)	-9.7 ± 2.1	-9.0 ± 2.1	-10.6 ± 2.1	0.813
LDL-C				
Start phase (mmol/L)	3.78 ± 0.13	3.70 ± 0.12	3.81 ± 0.13	
End phase (mmol/L)	3.21 ± 0.13[b]	3.14 ± 0.13[b]	3.11 ± 0.13[c]	0.398
Change (%)	-13.2 ± 3.0	-13.3 ± 3.0	-16.1 ± 3.0	1.000
HDL-C[1]				
Start phase (mmol/L)	1.36 ± 0.08	1.32 ± 0.07	1.37 ± 0.07	
End phase (mmol/L)	1.16 ± 0.05[a]	1.20 ± 0.05	1.14 ± 0.05[c]	0.370
Change (%)	-8.4 ± 2.5	-5.8 ± 2.5	-10.7 ± 2.5	0.218
TG[1]				
Start phase (mmol/L)	2.24 ± 0.22	2.31 ± 0.25	2.12 ± 0.21	
End phase (mmol/L)	2.34 ± 0.17	2.33 ± 0.17	2.41 ± 0.17	0.674
Change (%)	4.5 ± 7.3	0.8 ± 7.3	8.1 ± 7.5	0.651
Glucose				
Start phase (mmol/L)	5.30 ± 0.14	5.32 ± 0.14	5.28 ± 0.18	
End phase (mmol/L)	5.00 ± 0.08	5.00 ± 0.08[a]	5.03 ± 0.08	0.855
Change (%)	-1.2 ± 1.4	-3.2 ± 1.4	-0.2 ± 1.4	0.161

Abbreviations: WPF, whole pea flour; FPF, fractioned pea flour; WF, white wheat flour, TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides

Mean ± SEM, n=23.

Percent change is based on individual data

[a-c] Significantly different from day 1 & 2: [a]$p<0.05$, [b]$p<0.002$, [c]$p<0.001$

[§]Treatment effect for ANOVA across treatments

Statistical significance was set at $p < 0.05$

3.5 DISCUSSION

The novel findings presently demonstrate that under conditions that control for food and energy intake, the USDA's recommended dose of 50 g/day WPF (½ cup) and FPF, normalized to the fibre content in the WPF treatment, effectively reduced fasting insulin concentrations, and insulin resistance in hypercholesterolemic, overweight and obese individuals. In addition, women consuming WPF showed lower android-to-gynoid fat ratios compared to WF.

The observation that both pea treatments produced the same effect on fasting insulin concentrations suggests that pea fibre was the efficacious bioactive substance responsible for that action. It is likely that the effect of WPF and FPF on fasting insulin was secondary to a reduction in IR as indicated by the 25% in HOMA-IR, an estimate of IR.

The data suggest that less insulin was needed to maintain fasting glucose concentrations. Other studies have observed similar effects canned chickpeas (Pittaway et al., 2008) and cereal fibre (Weickert et al., 2006). Insulin resistance is considered a major risk factor for diabetes, CVD, hypertension, obesity and inflammation (Ginsberg, 2000; Watson et al., 2003). Moreover, whole yellow peas and fractionated yellow peas are high in insoluble fibre. A prospective cohort study demonstrated that diets rich in insoluble fibre reduced the risk of type 2 diabetes by 25% (de Munter et al., 2007). Similarly, a meta-analysis by Schulze et al revealed that cereal-derived fibre, a good source of insoluble fibre, was associated with a lower relative risk ratio for diabetes of 0.67 compared to soluble fibres from fruit and vegetables at 0.96 and 1.07, respectively (Schulze et al., 2007). Short chain fatty acid (SCFA) production in the gastrointestinal tract secondary to microbial fermentation of fibres has been shown to increase insulin sensitivity (Robertson, 2007; Robertson et al., 2005; Robertson et al., 2003; Thorburn et al., 1993). Researchers propose that SCFA reduce levels of circulating non-esterified fatty acids, a known inducer of insulin resistance (Savage et al., 2007). The notion of whole and

fractionated yellow peas evoking a prebiotic effect serves as a plausible mechanism to explain observed changes in insulin parameters. Why this effect occurred without acting on fasting glucose and/or post-prandial glucose responses cannot, however, be reconciled using the current data.

The present study attempted to define the effect of pea treatments on body composition in the context of an energy balanced paradigm. By preventing changes in body weight we demonstrated that WPF and FPF do not affect %TFM, independent of changes in body mass. Lack of effect on body composition was most likely due to controlling energy intake and maintaining body weight over the duration of each study phase. Furthermore, results from the present study suggest the WPF or FPF do not contain bioactive compounds that upregulate energy expenditure. Moreover, since subjects were required to consume all food provided by study personnel during each study phase, the negligible of effect on body composition with WPF and FPF treatments in the present study was observed in absence of pulses triggering satiety-related suppression of food intake; a potential mechanism of action of yellow peas, and ultimately pulses, on body composition (Delargy et al., 1997; Pittaway et al., 2008; Salas-Salvado et al., 2008).

Increased android adiposity is associated with CVD risk factors including insulin resistance, inflammation and atherosclerosis (Carey et al., 1996; Tanko & Christiansen, 2006). In the present study, women demonstrated a shift in fat distribution from android to gynoid with WPF, suggesting that bioactives other than fibre are responsible for the effects observed. WPF contains the cotyledon which houses various proteins and phytochemicals including secoisolariciresinol, a lignan-type phytoestrogen at 13 µg/100g (Mazur, 1998). Pea-derived secoisolarisciresinol is converted to enterolactone by colonic microflora and subsequently absorbed. While estrogen helps regulate regional fat deposition by interacting with estrogen receptor α (ERα) and β (ERβ) and upregulating α_2-adrenergic and β-adrenergic receptors, studies indicate that various phytoestrogens exhibit different affinities for ERα and ERβ. For example, soy-derived phytoestrogens

demonstrate ERβ binding specificity (Morito et al., 2001), while coumenstrol has been shown to be an ERα specific agonist (Morito et al., 2002; Wu et al., 2009). Although the biological effects of secoisolariciresinol are understudied, physiological levels of enterolactone were able to induce estrogen-dependent reporter gene expression via ERα receptors (Penttinen et al., 2007). Studies suggest that women express higher levels of subcutaneous ERα (Rodriguez-Cuenca et al., 2005) and α_2-adrenergic receptors (Richelsen, 1986) compared to men. Since estrogen favors subcutaneous fat deposition via ERα and α2-adrenergic receptors via lower lipolytic activity in subcutaneous adipose tissue (Pedersen et al., 2004; Richelsen, 1986), we hypothesize that pea-derived secoisolariciresinol could be acting as an ERα agonist in women through its conversion to enterolactone. Few studies have examined the effects of phytoestrogens on body composition. However, Sites et al.(2007) demonstrated that soy isoflavones reduced abdominal subcutaneous fat deposition post-menopausal women and suggested that the preferential affinity of soy-derived phytoestrogens for ERβ may explain their results. The observation that urinary enterolactone levels tended to be higher in the WPF group compared WF in present study augurs for further exploration of the effects of enterolactone on body fat distribution.

Failure for WPF and FPF to reduce fasting glucose concentrations is most likely secondary to the fact that most individuals were already normoglycemic with fasting glucose concentrations <5.6 mmol/L). Similar negative results have been observed with chickpea fortified bread products (Nestel et al., 2004) and high-fibre rye bread (Juntunen et al., 2003). WPF and FPF also failed to change post-prandial glucose responses when combined with an energetically balanced breakfast. Foods high in dietary fibre are thought to reduce post-prandial glucose responses by interfering with glucose absorption (Jenkins et al., 2000a). High levels of insoluble fibre found in yellow peas may have contributed to yellow peas inefficacy at reducing post-prandial glycemia since insoluble fibres are less effective than soluble fibres at blunting glycemic responses (Jenkins et al., 2000b). However, Guevin et al.(1996) noted that 10 g dietary fibre at a 1:4 and 2:3 ratio of soluble to insoluble fibre failed to produce any effect on post-prandial glycemia,

suggesting that factors other than fibre type are responsible for the varied results regarding the effect of pulses on glycemic response. It should be emphasized that in the present study, in order to eliminate the confounding effects of fibre from the diet, some fibre was removed from the background diet as breads were all composed of white flour. Hence, the caloric density and amount of simple carbohydrates of the breakfast meal may have been in excess for some subjects, such that the dose of WPF or FPF did not reduce glucose absorption. On the other hand, the calculated GI of the breakfast meal excluding treatment was low at ≈ 39.6 and was secondary to the high proportion of egg protein relative to carbohydrate content**.** Given that the GI of the breakfast meal was already reduced, post-prandial glucose concentrations may have been blunted such that the ¼ cup of peas during breakfast failed to exert any observable effect on glucose response. Finally, it has been suggested that traditionally cooked pulses, i.e. boiled, exert a greater effect on glucose response because of the lower surface area compared to equivalent amounts of pulse flour (Araya et al., 2003). Smaller particles, as found in flours, would have allowed greater surface exposure to digestive enzymes and hence a higher rate of absorption (Araya et al., 2002). Hence, using flours as a vehicle for administering WPF and FPF in the present study may have prevented a blunting in post-prandial glycemic response.

The observation that within-group reductions in lipid concentrations for TC, LDL-C and HDL-C did not translate into changes between treatment groups suggests alterations in fasting lipids were secondary to the NCEP-step 1 diet provided to volunteers during each treatment phase. Our results show no changes in lipid levels secondary to WPF and FPF treatments. Previous studies suggest that the dose of pulses used in the present study was insufficient to induce a lipid lowering effect. Fruhbeck et al. (1997) noted significant reductions in TC, LDL-C and TG with 90 g/day raw and cooked bean flour, a dose that was almost double the 50 g WPF given to volunteers in the present study. Conversely, Calvert et al. (1981) failed to observed a change in TC, LDL-C HDL-C and TG when subjects received 50 g/day defatted soybean flour for 28 days. The results from Calvert

et al.(1981), together with the present findings, suggest that the dose administered may have been insufficient to elicit a significant change in circulating lipid concentrations.

3.6 CONCLUSIONS

In summary, the present results suggest that the fibre content of ½ cup/day yellow peas (≈ 50 g) in the form of WPF and FPF improves CVD and diabetes risk factors by reducing fasting insulin concentrations and markers of insulin resistance independent of food and energy intake. The observation that women demonstrated a shift in subcutaneous fat deposition with WPF warrants further investigation into the phytoestrogenic effects of yellow peas.

3.7 ACKNOWLEDGEMENTS

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BRIDGE TO CHAPTER IV

Given the available literature demonstrating that pulse crops possess low glycemic indices, the data presented in Chapter III, showing no effect of either whole or fractionated yellow pea flour on post-prandial glycemia were unexpected. As outlined in Chapter III's Discussion section, we hypothesize that structural properties of pea flours and/or factors associated with the background breakfast meal explain the lack of effect of whole and fractionated pea flour on glycemia. Therefore, a study investigating the sole effects of novel foods formulated with pea-derived flours will determine their true hypoglycemic characteristics.

The purpose of the following study was to compare the post-prandial hypoglycemic effects of novel pulse-based food products formulated with whole yellow pea flour to white bread, boiled whole yellow peas and corresponding foods made with whole wheat flour. In addition, given that pulse crops possess sensory characteristics that can be undesirable, especially in North America, this study assessed and compared volunteers' perception of appearance, taste, smell and texture of each novel whole yellow pea flour food to corresponding whole wheat flour foods.

CHAPTER IV

GLYCEMIC RESPONSES AND SENSORY CHARACTERISTICS OF WHOLE YELLOW PEA FLOUR ADDED TO NOVEL FUNCTIONAL FOODS

Christopher P.F. Marinangeli[1], Amira N. Kassis[1], Peter J.H. Jones[1]

[1]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, 196 Innovation Drive, Winnipeg MB, R3T 2N2

To whom correspondence should be addressed:

Dr. Peter J.H. Jones PhD

Richardson Centre for Functional Foods and Nutraceuticals

University of Manitoba

196 Innovation Drive

Winnipeg, MB CANADA

R3T 2N2

Tel: +1 204 474 8883

Fax: +1 204 474 7552

Peter_jones@umanitoba.ca

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4.1 ABSTRACT

A fundamental understanding regarding post-prandial glycemic responses to foods containing whole yellow-pea flour (WYPF) remains unknown. This, alongside concerns that WYPF possesses unfavorable sensory characteristics has limited the incorporation of WYPF into new functional food products as a healthy novel ingredient. The objective of this study was to evaluate how WYPF modulates post-prandial glycemic responses as well as sensory characteristics in novel foods. In a single-blind cross-over trial, the present study assessed post-prandial glycemic responses of banana bread, biscotti and spaghetti containing either WYPF or whole wheat flour (WWF). Boiled yellow peas (BYP) and white bread (WB) were used as positive and negative controls, respectively. On d 1, subjects evaluated appearance, taste, texture, smell as well as overall acceptance of each WYPF and WWF food on a five point hedonic scale. WYPF banana bread (97.9 ± 17.8 mmol·min/L) and biscotti (83.0 ± 13.0 mmol·min/L), as well as BYP (112.3 ± 19.9 mmol·min/L), reduced ($p<0.05$) glycemic responses compared to WB (218.1 ± 29.5 mmol·min/L). The glycemic response of WYPF pasta (160.7 ± 19.4 mmol·min/L) was comparable to WB. WYPF biscotti produced a lower ($p=0.019$) post-prandial glycemic response compared to WWF biscotti (117.2 ± 13.1 mmol·min/L). Hedonic responses between corresponding foods were similar except for the WYPF pasta (2.9 ± 0.9) which possessed a lower sensory score ($p=0.02$) for smell compared to WWF pasta (3.6 ± 1.0). WYPF can be used to produce low-glycemic functional foods possessing sensory attributes that are comparable to identical food products containing WWF.

4.2 INTRODUCTION

Pulses, including yellow peas, are readily available, low-cost commodities that are gaining considerable momentum as functional ingredients possessing health benefits since they are high in fiber, protein, antioxidants, and low in fat (Champ, 2002; Leterme, 2002). Lifestyle interventions have been shown to be more effective in preventing type II diabetes compared to prescription medications (Knowler et al., 2002). As incidence rates of type II diabetes continue to climb, new novel ingredients that produce low-glycemic food products are in demand (Desrochers & Brauer, 2001). Consumption of whole pulses have been shown to lower post-prandial glycemic responses (Jenkins et al., 1983a), however, their addition to foods often produces unfavorable sensory characteristics. To date, few studies have examined the feasibility of producing food products containing pulse-derived ingredients that reduce post-prandial glycemic responses, lower glycemic indexes (GI) and at the same time possess sensory qualities that appeal to consumers. Since yellow peas are readily available and relatively inexpensive, they are a promising candidate for use as novel ingredients for the development of low-glycemic, consumer-friendly functional foods.

The objective of the present study was to first determine the post-prandial glycemic response and GI of novel foods containing whole yellow pea flour (WYPF) as a functional ingredient. The second objective was to determine the acceptability of sensory characteristics for each WYPF food product.

4.3 MATERIALS AND METHODS

4.3.1 Study Subjects

Twenty-two healthy men (n = 7) and non-lactating women (n = 15) between the ages of 22 and 67, and BMI's between 21 and 42 kg/m^2 were included in the study. Individuals with histories of heart disease, diabetes, thyroid disease, celiac disease or gluten intolerance, smokers, chronic alcohol use (> 2 drinks/d) or those taking corticosteroid medications were excluded from participating. Prior to initiating the study, subjects signed an informed consent form. The present study was approved by the University of Manitoba's Biomedical Research Ethics Board and is registered at clinicaltrials.gov, ID NCT00877968.

4.3.2 Food Products

Banana bread, biscotti, and pasta (spaghetti) treatment formulations are summarized in **Table 5**. Banana bread and biscotti were developed and prepared at the Richardson Centre for Functional Foods (RCFFN) clinical kitchen. Each food was prepared using either 100% WYPF or 100% whole wheat flour (WWF) as their primary ingredient. WYPF was provided by Best Cooking Pulses Inc. (Portage la Prairie, MB, Canada), while WWF was purchased locally. The test spaghetti was produced by the Canadian International Grains Institute (CIGI) using 30% WYPF, while the balance of the flour weight was white wheat durum. Whole wheat spaghetti was purchased locally and produced with 100% whole wheat durum. All spaghettis were prepared at the RCFFN clinical kitchen.

4.3.3 Assessment of Post-prandial Glucose Response and Glycemic Index

Using a single-blind crossover design, each subject was randomly assigned to a different intervention sequence which determined the order whereby each treatment would be administered. Study subjects arrived at the RCFFN in the morning following a 12 hr over-night fast. Doses for each food product were standardized such that each serving provided 50 g of available carbohydrate (Table 5). Subjects also consumed 250 ml of water with each test product. Subjects were given 30 min to eat each food product. Blood glucose levels were tested again 30, 60, 120 and 150 min after the start of the meal. Fasting glucose levels were evaluated using finger prick glucose monitors (Accu-Chek plus, Roche diagnostics, Laval, QC., Canada). For each glucose test, two blood readings were taken and averaged. If blood glucose levels were > 0.3 mmol apart, a third reading was taken. A two day washout period was implemented between each glycemic response test.

Incremental areas under the curve (IAUC) were calculated using methods described by Wolever et al. (1991). Briefly, areas below baseline values were subtracted from total area under the curve. If blood glucose values fell below baseline, IAUC at those data points were also subtracted from the total. Glycemic indexes (GI) were calculated by dividing the IAUC for the test food by the IAUC of WB and multiplying the ratio by 100 (Wolever & Jenkins, 1986).

Table 5. Nutritional composition of treatment flours and food products formulated with treatment flours

	Flours		Post-prandial Glucose Response Servings					
			Banana Bread		Biscotti		Pasta (dry)	
	WYPF[§φ]	WWF[¥]	WYPF	WWF	WYPF	WWF	WYPF (30%)[¶]	WWF
Serving (g)	100.0	100.0	67.8	62.8	86.0	79.8	90.0	70.0
Energy (KJ)	1120.9	1418.4	1543.5	1522.6	1502.5	1458.1	1133.9	1068.2
Carbohydrate (g)	45.3	72.6	52.0	51.7	51.7	53.2	51.1	51.1
Total fiber (g)	17.6	12.2	8.1	7.1	10.1	8.2	8.1	6.6
Soluble (g)	2.0	2.1	3.0	3.1	3.3	3.3	3.4	NA
Insoluble (g)	15.6	10.1	5.1	4.0	6.4	4.4	4.8	NA
Fat (g)	1.6	1.9	15.2	16.1	13.3	13.3	1.6	1.2
Protein (g)	19.2	13.7	9.3	7.8	12.2	9.5	7.4	9.9

Abbreviations: WYPF, whole yellow pea flour; WWF, whole wheat flour; NA not available

[§] WYPF used to manufacture all WYPF treatments.

[φ] Analysis of total, soluble and insoluble fibre in WYPF was completed by Medallion Labs using AOAC method 991.43.

[¥] WWF used to manufacture WWF banana bread and WWF biscotti

[¶] WYPF pasta was 30% WYPF with the balance being white wheat flour

4.3.4 Assessment of Sensory Characteristics

The subjects who participated in the post-prandial glucose response tests also evaluated the sensory characteristics of the food products. Prior to d 1, subjects visited the University of Manitoba's Weston Food Sensory Lab. Study personnel described how the sensory analysis would ensue as well as the hedonic scales that would be used to rate each food product. On d 1 of the study, subjects were seated in a private booth to prevent interaction with other study volunteers. Samples of each food were provided one at a time. Prior to receiving subsequent food samples, previous products were removed and volunteers cleansed their pallet with water. For each food product volunteers completed a questionnaire assessing appearance, taste, smell, texture as well as overall acceptance. Each parameter was evaluated on a 5 point hedonic scale (Dislike very much = 1, Dislike = 2, Neither like nor dislike = 3, Like = 4, Like very much = 5). Water was given to volunteers between test foods. Scores for each product were compiled for statistical analysis.

4.3.5 Statistical Analysis

Comparisons of IAUC and GI between WYPF products, BYP and WB were analyzed using mixed model analysis of covariance (ANCOVA) using subject as a random variable and baseline glucose as a covariate. Differences between groups were identified using the Bonferroni post hoc test. Differences in IAUC and GI between corresponding food products were analyzed using paired t-tests. All glycemic data are presented as mean ± SEM. Comparisons regarding hedonic sensory analysis scores between corresponding food products were analyzed using paired t-tests and presented as mean ± SD. A level of significance at $p<0.05$ was used in all statistical analyses. Data were analyzed using SPSS software version 11.5 (SPSS Inc. Chicago, Illinois, USA).

4.4 RESULTS AND DISCUSSION

4.4.1 Oral Glucose Tolerance Tests: Incremental Area under the Curve and Glycemic Index

Nineteen of the 22 subjects recruited completed glycemic response testing. Three female volunteers dropped out because of time commitment restrictions. Comparisons of post-prandial glucose responses for WYPF products, BYP and WB are summarized in **Figure 4.** Post-prandial glucose responses comparing WYPF products to corresponding foods made with WWF are summarized in **Figure 5A-C**. Results show IAUC values for WYPF biscotti (83.0 ± 13.0 mmol·min/L) and WYPF banana bread (97.9 ± 17.9 mmol·min/L) were 61.9% and 55.1% lower ($p<0.001$) compared to WB (218.1 ± 29.5 mmol·min/L), respectively (**Table 6)**. Conversely, IAUC for WYPF pasta (160.7 ± 19.4 mmol·min/L) was 43.1% higher ($p=0.019$) than BYP (112.3 ± 19.9 mmol·min/L). Paired T-tests between corresponding WYPF and WWF products indicate no differences in IAUC values for banana bread products and pasta products (**Table 7)**. IAUC for WYPF biscotti was 29.2% lower ($p=0.019$) than WWF biscotti (117.2 ± 13.1 mmol·min/L).

Calculated GI values of WYPF products are provided in **Tables 6 & 7**. WYPF biscotti (45.4 ± 8.2) *($p<0.001$)*, WYPF banana bread (50.3 ± 8.0) ($p=0.013$) and BYP (59.9 ± 11.9) ($p=0.007$) have lower calculated GIs compared to whole pea pasta (93.3 ± 9.4). Paired t-tests indicate that WYPF biscotti elicited a lower ($p=0.013$) GI compared to WWF biscotti (63.9 ± 7.1) (**Table 7**).

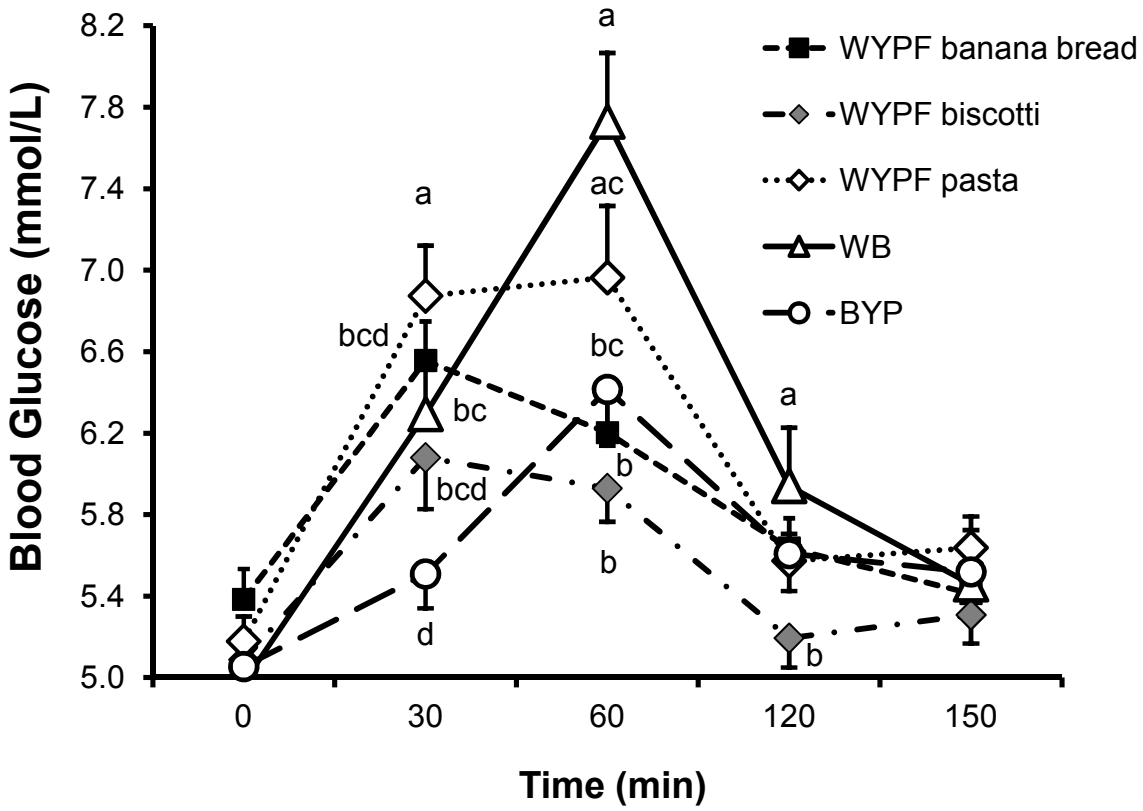

Figure 4. Glycemic response curve for WYPF banana bread, WYPF biscotti, WYPF pasta as well as BYP and WB. Abbreviations: WYPF, whole yellow pea flour; WB, white bread; BYP, boiled yellow peas. Different letter subscripts indicate values that are significantly different from each other within each time point *($p<0.05$)* (n=19).

Factors identified as capable of reducing post-prandial glucose responses include total amount and types of fibre present in test foods (Jenkins et al., 2000a), protein (Frid et al., 2005) and fat content (Collier & O'Dea, 1983), starch-protein interactions (Jenkins et al., 1987), as well as the presence of anti-nutrients such as phytic acid, phenols and tannins (Champ, 2002). Factors responsible for WYPF improving glycemic responses in WYPF biscotti and banana bread remain difficult to delineate from the current data.

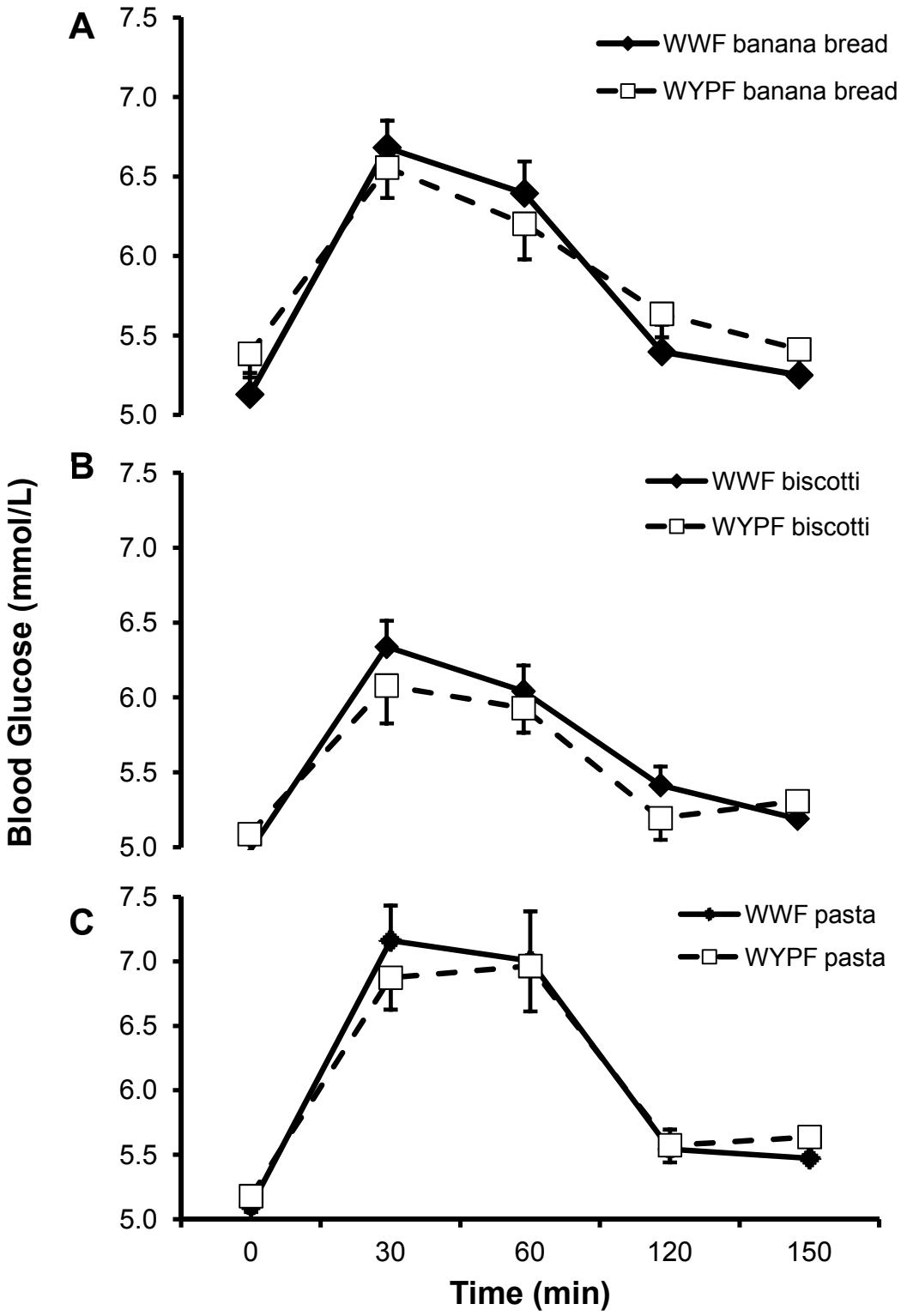

Figure 5 A-C. Glycemic response curves comparing WYPF banana bread with WWF banana bread (A), WYPF biscotti and WWF biscotti (B) and WYPF pasta and WWF pasta (C). (n=19).

The present study contradicts previous research that suggests pulse-derived flours in bread products do not induce the same reductions in post-prandial glucose response as do traditionally cooked (boiled), pulses. Studies evaluating *in* vitro digestibility of flours have suggested that small particle sizes of flour granules allow digestive enzymes to liberate carbohydrate that would not otherwise be absorbed (Hawkins & Johnson, 2005). Conversely, comparison of WWF and an ultra-fine WWF revealed that particle size does not necessarily influence glycemic responses in humans (Behall et al., 1999), supporting the results of the present study.

Table 6. IAUC and GI between WYPF products, BYP and WB

Response	WYPF			BYP	WB
	Banana Bread	Biscotti	Pasta		
IAUC[§]	97.9 ± 17.8^{a}	83.0 ± 13.0^{a}	160.7 ± 19.4^{b}	112.3 ± 19.9^{a}	218.1 ± 29.5^{b}
GI	50.3 ± 8.0^{a}	45.4 ± 8.2^{a}	93.3 ± 9.4^{b}	59.9 ± 11.9^{a}	-

Abbreviations: BYP, boiled yellow peas; GI, glycemic index; IAUC; incremental area under the curve; WYPF, whole yellow pea flour; WB, white bread

All values are mean ± SEM, n=19

Differences between food products were determined mixed model ACNOVA

[§] Units for IAUC values are mmol·min/L

Values with different letter superscripts are significantly different at $p<0.05$

Table 7. IAUC and GI between corresponding food products

Response	Banana Bread			Biscotti			Pasta		
	WYPF	**WWF**	***p-value*[¥]**	**WYPF**	**WWF**	***p-value*[¥]**	**WYPF**	**WWF**	***p-value*[¥]**
IAUC[§]	97.9 ± 17.8	126.1 ± 16.5	0.233	83.0 ± 13.0[a]	117.2 ± 13.1[b]	0.019	160.7 ± 19.4	179.4 ± 19.8	0.290
GI	50.3 ± 8.0	75.4 ± 14.9	0.097	45.4 ± 8.2[a]	63.9 ± 7.1[b]	0.013	93.3 ± 9.4	83.6 ± 9.6	0.365

Abbreviations: GI, glycemic index; IAUC; incremental area under the curve; WYPF, whole yellow pea flour; WWF, white wheat flour

All values are mean ± SEM, n=19

Differences between corresponding food products were determined using paired t-tests

[§] Units for IAUC values are mmol·min/L

[¥]Values with different letter superscripts are significantly different at $p<0.05$

A potential mechanism as to why the WYPF pasta showed no effect of post-prandial glycemia could be related to the dosage of pulse-derived flour added to food products (**Table 6**). In the present study the WYPF pasta was only 30% WYPF which may have been insufficient to blunt the glycemic response considering the balance of the flour was white wheat durum. Similarly, Johnson and others (2005) showed that bread containing 24.3% chickpea flour failed to reduce overall IAUC or GI compared to WB. Since wheat was the foremost ingredient in both WWF and WYPF pastas, degree of starch gelatinization is another plausible explanation for WYPF pasta's lack of effect on glycemic response. The combination of spaghetti's large surface area, high cooking temperatures and available water while boiling, provided ideal conditions for the swelling of starch granules and improved starch hydrolyzation by digestive enzymes (Holm et al., 1989; Holm et al., 1988). Depending on processing methods, pulses have been shown to be less susceptible to starch gelatinization (O'Dea & Wong, 1983; Wursch et al., 1986). In addition, water was not an ingredient for the biscotti and banana bread treatments, and neither bread-based treatment was exposed to water during cooking. Hence, gelatinization would not occur to the same degree, preventing subsequent enzymatic hydrolyzation of starch during digestion. In fact, our results clearly show higher efficacy of banana breads and biscottis versus pastas, regardless of the flour component used. In addition, the extrusion and industrial drying processes used to produce dry pasta, as well as exposing the pasta's larger surface area to boiling water, may have destroyed anti-nutritional factors such as phytates and polyphenols (Bishnoi et al., 1994; Estevez et al., 1991; Sandberg, 2002) which have been shown to inhibit glucose absorption (Alonso et al., 2000; Thompson et al., 1984). Conversely, the yellow peas in the WYPF used to produce the bread products were dried while still on the vine and did not undergo any thermal or pressurized processing procedures prior to cooking. Lower surface area exposure of WYPF to direct heat may have also played a role in preserving levels of anti-nutrients in the baked products, since they were cooked as whole loaves. Overall, considering the aforementioned factors and their potential interactions, we suggest that

Table 8. Comparison of sensory characteristics between similar foods produced using WWF or WYPF

Sensory Attribute	Banana Bread			Biscotti			Pasta		
	WYPF	**WWF**	***p-value*[§]**	**WYPF**	**WWF**	***p-value*[§]**	**WYPF**	**WWF**	***p-value*[§]**
Appearance	4.0 ± 0.8	4.0 ± 0.7	0.833	3.2 ± 1.0	3.2 ± 1.2	0.834	3.8 ± 0.5	3.8 ± 0.7	0.789
Smell	4.0 ± 0.8	4.0 ± 0.7	0.665	3.5 ± 0.9	3.5 ± 0.9	0.825	2.9 ± 0.9[a]	3.6 ± 1.0[b]	0.020
Taste	3.8 ± 0.8	4.0 ± 1.1	0.397	3.3 ± 1.2	3.4 ± 1.2	0.741	3.5 ± 0.9	3.8 ± 0.8	0.267
Texture	4.1 ± 0.6	4.2 ± 0.7	0.789	2.6 ± 1.3	3.1 ± 1.1	0.077	3.5 ± 0.9	3.8 ± 0.7	0.248
Overall	4.0 ± 0.7	4.1 ± 0.8	0.544	3.1 ± 1.1	3.4 ± 1.1	0.111	4.6 ± 0.8	3.9 ± 0.8	0.348

Abbreviations: WYPF, whole yellow pea flour; WWF, White wheat flour

All values are mean ± SD, n=22

Differences between corresponding food products were determined using paired t-tests

[§]Values with different letter superscripts are significantly different set at $p<0.05$

the food matrix is an important determinant as to whether WYPF produces a low glycemic food.

4.4.2 Sensory Analysis

All 22 subjects completed sensory assessments of WYPF and WWF foods. Results comparing sensory characteristics of corresponding foods are summarized in **Table 8**. All food products scored higher than 3.0 (Neither like nor dislike) for all categories except for WYPF biscotti (2.6 ± 1.3) for "texture" and WYPF pasta (2.9 ± 0.2) for "smell". The only sensory differences noted were for "smell," whereby acceptability for WYPF pasta was 9.4% lower ($p=0.02$) than WWF pasta (3.2 ± 0.2).

Sensory analysis revealed that all products formulated with WYPF were comparable to corresponding foods produced using WWF, except for pasta. WYPF pasta's lower score for smell compared to WWF suggests that the 70% white wheat flour in the WYPF pasta lacked the pungent sensory qualities necessary for masking unpleasant odors often associated with traditional pulse and pulse-derived food products. Conversely, unpleasant sensory traits associated with WYPF were easier to manipulate in the baked products through the use of secondary ingredients that possess favorable sensory characteristics.

4.5 CONCLUSIONS

WYPF can be used as a functional ingredient to produce novel low-glycemic foods with favorable sensory characteristics. The observation that neither WYPF pasta, nor the corresponding WWF pasta, showed any difference in glycemic response compared to WB, suggests processing and cooking factors rather than the WYPF itself, contributed to WYPFs lack of efficacy within the pasta matrix. Similar sensory characteristic scores

between WYPF biscotti and banana bread compared to corresponding products formulated with WWF indicate that supplemental ingredients having prominent sensory qualities should be added to pulse-derived products to improve acceptance amongst consumers. Results from the present study support the use of WYPF as a tool for health care practitioners to assist patients in cooking tasty, low-glycemic foods that help prevent and manage type II diabetes.

4.6 ACKNOWLEDGMENTS

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BRIDGE TO CHAPTER V

Few studies have examined the effects of pulse crops on energy expenditure. Given their high levels of protein and fibre, studies suggest that pulse crops could increase or decrease post-prandial energy expenditure, respectively. The discovery that whole and/or fractionated yellow pea flours are thermogenic food ingredients would facilitate the development novel food products that enhance weight loss. Conversely, reductions in energy expenditure would also yield food products with clinically relevant applications. For example, individuals coping with disease states that facilitate weight loss would benefit from foods that lower post-prandial thermogenesis since less energy would be utilized for digestion. Finally, given that yellow peas are excellent sources of insoluble fibres and recent animal studies demonstrate that dietary insoluble fibres modulate the expression of transcription factors that upregulate lipid β-oxidation, exploratory studies that investigate pulses' effect on lipid oxidation and hepatic triglyceride synthesis are warranted.

The purpose of the following study was to determine if whole or fractionated yellow pea flours, at USDA recommended dosages, modulate post-prandial energy expenditure, thermic effect of food and substrate utilization. In addition, Chapter V aims to delineate whether whole or fractionated yellow pea flours modulate dietary fat oxidation and hepatic triglyceride synthesis.

CHAPTER V

CHRONIC INTAKE OF FRACTIONATED YELLOW PEA FLOUR REDUCES POST-PRANDIAL ENERGY EXPENDITURE AND CARBOHYDRATE OXIDATION

Christopher P.F. Marinangeli[1] and Peter J.H. Jones[1]

[1]Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, 196 Innovation Drive, Winnipeg MB, R3T 2N2, Canada

To whom correspondence should be directed:

Peter J.H. Jones, PhD

University of Manitoba

Richardson Centre for Functional Foods and Nutraceuticals

196 Innovation Drive

Winnipeg, MB Canada

R3T 2N2

Tel: +1 204 474 8883

Fax: +1 204 474 7337

Peter_jones@umanitoba.ca

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5.1 ABSTRACT

Effects of dietary fibres on human post-prandial energetics remain undefined. The objective of the present study was to explore effects of whole yellow pea flour (WPF) and fractionated pea flour (FPF) on post-prandial energy expenditure, substrate-utilization, and hepatic triglyceride synthesis rate. Using a crossover-diet controlled design, 23 overweight men and women received muffins containing WPF, FPF and white flour (WF) for 28 d, followed by 28 d washout periods. Subjects received 50 g/d WPF and WF. Given that FPF is ≈84% fibre, the amount of FPF administered to volunteers was equivalent to the amount of pea-derived fibre in the WPF treatment. Four weeks of FPF consumption reduced (*p=0.007)* total post-prandial energy expenditure (333.0±3.6 kcal/330 min) compared to WF (349.3±3.6 kcal/330 min). When normalized to the level of food energy consumed, FPF (4.6±0.3%) decreased (*p=0.018*) the thermic effect of food (TEF) compared to WF (5.7±0.3%). Carbohydrate oxidation tended to be lower (*p=0.075*) with FPF (44.7±2.1 g/330 min) versus WF (51.2±0.1.9 g/330 min). WPF had no effect on total energy expenditure, TEF or carbohydrate oxidation. Only after 370 min was cumulative oxidation of 1-^{13}C-palmitic acid higher (*p=0.*045) in the WPF group (0.96±0.05%) compared to FPF (0.81±0.05%). Neither treatment had any effect on hepatic triglyceride synthesis rate. The present study demonstrated that chronic intake of FPF decreased post-prandial energy expenditure, carbohydrate oxidation and TEF compared to WF, while WPF showed no effect on post-prandial energetics. In conclusion chronic ingestion of different fractions of yellow peas imposes distinctive effects on post-prandial energy expenditure and substrate utilization.

5.2 INTRODUCTION

Despite considerable interest regarding the effects of certain pulse crops on circulating lipids and post-prandial glycemic response, little research has explored how pulse containing foods impact post-prandial energy expenditure and macronutrient oxidation in healthy humans. Touted as "superfoods," pulse crops including lentils, chickpeas, beans and peas, are relatively inexpensive, low in fat and high in protein, fibre, and antioxidants (Leterme, 2002). In theory the protein content of pulse foods may increase total energy expenditure after a mixed meal since protein has been shown to induce the highest thermic effect of food (TEF) compared to fat and carbohydrate (Johnston et al., 2002; Robinson et al., 1990). Conversely, high fibre meals have been shown to induce the opposite effect on energy expenditure (Scalfi et al., 1987). In the wake of the current obesity epidemic, evidence that pulse crops can modulate energy dynamics after a given meal could foster further weight-loss or reductions in post-prandial adipogenesis when utilized in conjunction with other lifestyle modifications.

Currently the USDA recommends that ½ cup pulses be consumed daily as part of a healthy diet (USDA.gov, 2009). However, recommended levels of intake are difficult to attain since, compared to wheat, pulse crops are rarely utilized domestically and by the food industry as ingredients for formulating novel food products. Poor variety alongside unfavorable sensory characteristics that are unappealing to certain populations, especially in North America, have limited extensive integration of pulse crops into foods (Desrochers & Brauer, 2001). However, with the development of pulse-derived flours, novel functional foods that utilize pulses as the primary ingredient can be seamlessly integrated into diets devoid of pulse crops.

Although whole yellow peas (*Pisum sativum L.*) are one of the most widely cultivated pulse crops, the health benefits of whole yellow peas and their fractions remain largely undefined. Given that yellow peas provide a good source of protein and fibre, their incorporation into food products may increase or decrease post-prandial energy expenditure. Investigating and identifying the thermogenic effects of functional foods that incorporate whole and fractionated yellow peas as the foremost ingredient, could provide cost-effective options for harnessing the health benefits associated with pulse consumption.

The objectives of the present study were to investigate the effects of whole yellow pea flour (WPF) or fractionated yellow pea flour (FPF) on post-prandial energy expenditure, substrate utilization, meal-derived fat oxidation and hepatic triglyceride (TG) synthesis.

5.3 MATERIALS AND METHODS

5.3.1 Subjects

Twenty-nine hypercholesterolemic, overweight men (BMI 25 to 40 kg/m^2) and women (BMI 24 to 38 kg/m^2) residing in the city of Winnipeg were recruited using radio and newspaper advertisements. Baseline characteristics of study subjects are summarized in **Table 9.** Exclusion criteria included smoking, use of prescription and natural lipid lowering therapies, history of myocardial infarction, coronary artery bypass, angina, congestive heart failure, inflammatory bowel disease, pancreatitis, renal disease, diabetes and a history of chronic alcohol usage (> 2 drinks/d). Individuals with cancer, eating disorders and those who reported expending over 4000 kcal/wk through exercise were also excluded from participation. Participants taking medications for other ailments were included providing their daily dose of medication remained stable for the duration of the study. Prior to initiating the study, all subjects read and signed an informed consent

form. This study was approved by the University of Manitoba's Biomedical Research Ethics Board. This study was registered at www.clinicaltrials.gov, ID NCT00839774 (February 6, 2009).

Table 9. Baseline characteristics of study subjects

Criteria	Men n = 10	Women n = 19
Age	51.8 ± 12.3	52.3 ± 10.0
Weight (kg)	95.0 ± 18.6	80.4 ± 12.1
BMI (kg/m^2)	31.7 ± 5.3	29.4 ± 3.5
TC (mmol/L)[§]	5.63 ± 0.53	6.26 ± 0.81
LDL-C (mmol/L)[§]	3.32 ± 0.96	3.92 ± 0.75
HDL-C (mmol/L)[§]	1.11 ± 0.14	1.52 ± 0.33
TG (mmol/L)[§]	2.52 ± 1.31	1.85 ± 1.04
Glucose (mmol/L)[§]	5.79 ± 1.32	4.84 ± 0.49

[§] Fasted levels in serum

Abbreviations: TC, total cholesterol, LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides

Mean±SD

5.3.2 Treatments

Treatments consisted of WPF, FPF (hulls only) and white wheat flour (control) (WF). Treatment flours were administered to subjects in muffins. Muffin recipes were developed at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) clinical kitchen. WPF and FPF flours were obtained from Best Cooking Pulses Inc. (Portage la Prairie, MB, Canada), while the WF was obtained from a local supermarket. Pea treatment flours were analyzed by Medallion Labs (Minneapolis, MN, USA) to

determine their nutrient composition (**Table 10**). The composition of WF was determined by available food analysis tables (Brault Dubuc & Caron-Lahaie, 1994). Current USDA recommendations for pulse consumption are ½ cups/d, which is ≈ 50 g dry whole yellow peas (USDA.gov, 2009). Given that each WPF treatment muffin was formulated to contain ≈ 25 g WPF, subjects were required to eat two treatment muffins/d. The FPF treatment muffins were formulated based on total pea-derived fibre content of

the WPF muffin since the pea hull flour used in the present study was approximately 84.2 % fibre (**Table 10**). Hence, the amount of fractionated flour added to the FPF muffin recipe was adjusted to equal the amount of fibre in the WPF treatment (**Table 10**). The balance of the muffin weight was white wheat flour. The WF muffin was solely comprised of white wheat flour.

5.3.3 Study Design

The present study was a triple crossover, controlled-diet design with three 28 d study phases, each followed by a 28 d washout. In addition, the present study was single-blinded whereby volunteers were not informed as to which treatment they were receiving during a given treatment phase. To determine the treatment order within each subject, participants were randomly assigned to one of the six possible treatment sequences.

Table 10. Nutritional composition of treatment flours and treatment muffins

Treatment Flour	**WPF**[1§]	**FPF**[1§]	**WF**[2]
Energy (kJ)/100 g	1 281	343	1 482
Total carbohydrate (%)	66.3	89.0	79.4
Available carbohydrate (%)	50.7	12.7	76.3
Fibre (%)	17.6	84.2	3.1
Soluble (%)	2.0	7.9	1.8
Insoluble (%)	15.6	76.3	1.3
Total fat (%)	1.84	0.63	1.0
Polyunsaturated fat (%)	0.98	0.31	0.41
Monounsaturated fat (%)	0.44	0.13	0.09
Saturated (%)	0.34	0.16	0.15
Protein (%)	21.6	6.39	10.3
Treatment Muffins	**WPF**[1]	**FPF**[1]	**WF**[2]
Whole pea flour per muffin (g)	26.4	-	-
Fractionated pea flour per muffin (g)	-	6.0	-
White wheat flour per muffin (g)	-	20.4	26.4
Weight per muffin (g)	91	91	91
Energy (kJ)	821.5	816.9	888.5
Carbohydrate (g)	30.4	33.8	33.0
Total fibre (g)	5.8	6.9	2.0
Soluble fibre (g)	1.7	2.0	1.6
Insoluble fibre (g)	4.1	4.8	0.3
Total fat (g)	7.4	7.1	7.1
Polyunsaturated fat (g)	3.8	3.7	3.7
Monounsaturated fat (g)	3.2	3.1	3.1
Saturated fat (g)	0.9	0.8	0.8
Protein (g)	7.1	3.9	4.1

Abbreviations: Abbreviations: WPF, whole pea four; FPF, fractionated pea flour; WF, white wheat flour

[1] Treatment flours analyzed at Medallion Labs (Minneapolis, Minnesota, USA)

[§] Analysis of total, soluble and insoluble fibre was completed by Medallion Labs using AOAC method 991.43.

[2] Flour composition determined using available nutritional tables (Brault Dubuc and Caron-Lahaie, 1994)

5.3.3.1 Diet and Treatment Protocol

For the duration of each study phase, subjects were instructed to consume only food provided by the RCFFN clinical research kitchen. The nutrient composition of the study diet was analyzed using Food Processor version 7.71 (ESHA Research, OR, USA). The background diet had a nutrient composition of 55% carbohydrate, 15% protein, and 30% fat. Diets were designed to contain no pulse products besides the treatment muffins. To prevent bodyweight change for the duration of each study phase, daily energy requirements for each subject were determined by calculating individual resting metabolic rates using the Mifflin Equation (Mifflin et al., 1990), then applying an activity factor of 1.7. The amount of food each participant received was based on individual calculated energy requirements. The average caloric content of two treatment muffins (approximately 400 kcal) was subtracted from the total calculated energy requirement to compensate for the energy intake from the treatments. Body weights were assessed daily. If a subject's weight increased or decreased during the first week of each study phase, energy intake was adjusted accordingly. Each morning, study participants were required to visit the RCFFN Clinical Research Unit to consume breakfast and one treatment muffin under supervision. Participants would then leave the unit with a cooler containing the remainder of their meals and snacks alongside a treatment muffin to consume with dinner. Empty, unwashed food containers were used to monitor compliance.

5.3.3.2 Energy Expenditure Protocol

On a single day during the first and last week of each study phase, energy expenditure was determined using open circuit indirect calorimetry (Vmax Encore, Summit Technologies Inc, Burlington ON Canada) fitted with a ventilated canopy. The flow sensor and gas sensors were calibrated daily prior to initiating respiratory measurements. The flow sensor was calibrated using a calibration syringe. Gas sensors were automatically calibrated by Vmax Encore software (Summit Technologies Inc.,

Burlington ON Canada) using two reference gasses with the first containing 16% O_2, 4% CO_2 and 80% N_2 and the second containing 26% O_2, 0% CO_2 and 74% N_2. All indirect calorimetry data was collected using Vmax Encore software. Finally, commencement of pre-menopausal women into the study was planned such that they did not undergo indirect calorimetry measurements during their menstrual cycle.

After a 12 hr fast, subjects arrived at the RCFFN. After being allowed to rest for a period of 20 min, subjects were comfortably installed in a bed in a supine position. The ventilated hood was placed over the subject's head and resting energy expenditure (REE) was measured for 30 min prior to their breakfast meal. Subjects were given 40 min to consume their calorically controlled breakfast accompanied by one treatment muffin. In addition, the breakfast corresponding to day 1 of the three-day menu cycle was always consumed on the day that subjects underwent energy expenditure measurement (**Table 11**). Volunteers were instructed to consume the treatment muffins prior to ingesting any other foods in the breakfast meal. Post-prandial energy expenditure was measured in 30 min increments immediately following breakfast and again 60, 120, 180, 240, and 300 min after the breakfast meal. For all measurements, subjects remained motionless in bed in the supine position. At times when energy expenditure was not being measured subjects were required to remain in the supine position. Washroom breaks were permitted during the break periods.

Table 11. Diet composition of the breakfast meal consumed during energy expenditure measurements

100% pure Orange Juice
2% Milk
Corn flakes
Omelet with tomatoes
Bagel with Strawberry Jam
Non-hydrogenated Margarine
Treatment muffin

For each time increment, only respiratory gas exchange values recorded during the last 15 min of each measurement period were used to calculate total energy expenditure (kcal/min), carbohydrate (CHO) oxidation (g/min) and fat oxidation (g/min). Total energy expenditure was calculated using equations described by Lusk (Lusk, 1924).

$$TEE\ \left(\frac{kcal}{min}\right) = 4.686\ +\ \left(\left(\frac{npRQ - 0.707}{0.293}\right) \times 0.361\right)$$

Where 4.686 represents the kcal/L O_2 consumed with 100% fat oxidation, 0.707 is the respiratory quotient (RQ) $\left(RQ = \frac{VCO_2}{VO_2}\right)$ corresponding to 100% fat oxidation, 0.293 corresponds to the difference in RQ between 100% CHO oxidation and 100% fat oxidation, and 0.361 is the difference in Kcal/L O_2 between 100% CHO oxidation and 100% fat oxidation. Non-protein RQ (npRQ) was calculated based on the assumption that nitrogen is excreted at a constant rate of 0.14 g/kg body weight/24hrs (Jones & Schoeller, 1988):

$$npRQ = \frac{VCO_2 - \left(\left(body\ weight \times \frac{0.14}{1440}\right) \times 6.03\right)}{V0_2 - \left(\left(body\ weight \times \frac{0.14}{1440}\right) \times 4.88\right)}$$

Where 6.03 and 4.88 represent the volume (L) of CO_2 and O_2 consumed per gram of nitrogen metabolized (Westenskow et al., 1988). CHO and fat oxidation were calculated using the following equations:

$$CHO\ OX\ \left(\frac{g}{min}\right) =\ npVO_2 \times \left(\frac{npRQ - 0.707}{0.293 \times 0.746}\right)$$

$$Fat\ OX\ \left(\frac{g}{min}\right) =\ npVO_2\ \times \left(\frac{1.00 - npRQ}{0.293 \times 2.019}\right)$$

Where 0.746 and 2.019 represent the volume of O_2 (L) consumed per g of glucose and triglyceride oxidized, respectively (Jequier et al., 1987; Schutz, 1995).

Incremental areas under the curve (AUC) were calculated for each time interval using the trapezoidal rule. Total energy expenditure, total CHO and fat oxidation represented the total AUC. Thermic effect of food (TEF) was calculated by subtracting the area below REE from the total AUC.

5.3.3.3 Determination of Dietary Fat Oxidation

During week four, dietary fat oxidation was assessed on the same day as the indirect calorimetry session. Immediately following the measurement of REE, a baseline breath sample was collected into 12 ml evacuated glass tubes (Labco Exetainer®, Buckinghamshire, UK) using breath collection bags fitted with a gas collection port (EasySampler™, Quintron Instrument Co., Milwaukie, WI). During the breakfast meal, subjects consumed warm margarine containing 1-^{13}C-palmitic acid (Cambridge Isotope Laboratories Inc. CAS57677-53-9, Andover, MA) at 10mg/kg body weight which was spread over the treatment muffin. Additional breath samples were taken immediately following the breakfast meal, and again at 30, 90, 150, 210, 270, and 330 min following each indirect calorimetric session.

Breath samples were analyzed using continuous flow isotope ratio mass spectrometry (IRMS) (ABCA, SerCon Ltd., Cheshire, UK) and analyzed using ABCA breath analyzer software version 500.1.12 (SerCon Ltd., Cheshire, UK). The IRMS was calibrated against a reference gas containing 5% CO_2. Samples were normalized against Pee Dee Belemnite limestone (PDB) where $\frac{^{13}C}{^{12}C} = 0.0112372$. ^{13}C enrichment in the sample was calculated as:

$$\delta^{13}C(‰) = \frac{(R_{sa} - R_{pdb})}{R_{pdb}}$$

Where, δ^{13}C represents parts per thousand $\frac{^{13}C}{^{12}C}$ of the sample relative to R_{pdb}, R_{sa} is the $\frac{^{13}C}{^{12}C}$ ratio in the sample, and R_{pdb} is the constant ratio of PDB (Slater et al., 2001). Atom percent ($AP^{13}C$) was calculated as (Slater et al., 2001):

$$AP^{13}C = \frac{100}{\frac{1}{\left(\frac{\delta^{13}C}{100} + 1\right) \times {}^{13}R_{pdb}} + 1}$$

Atom percent excess (APE) was calculated as the difference between $AP^{13}C$ at each time interval and $AP^{13}C$ at baseline (Slater et al., 2001). Percent dose recovered per min (PDR) was determined using the following equation (McCloy et al., 2004):

$$PDR = \frac{APE \times mmol\ CO2\ expired\ per\ min}{mmol\ {}^{13}C\ administered} \times 100\%$$

Where mmol CO_2 expired per min was calculated multiplying mmol CO_2= 22.7 mmol/L by the volume of CO_2 expired per min as determined by indirect calorimetry (McCloy et al., 2004). The amount of ^{13}C administered (mmol) was calculated using the following formula (McCloy et al., 2004):

$$mmol\ {}^{13}C = \left[\left(\frac{mg\ 16{:}0\ administered}{molecular\ mass\ of\ 16{:}0}\right) \times chemical\ purity\ (\%)\right] \times$$

$$[(0.99 \times \#\ of\ {}^{13}\text{C}\ labelled\ Carbons) + (0.01 \times total\ \#\ of\ Carbons)\ ]$$

Where 0.99 accounts for 99% labeling efficiency of 1-^{13}C in administered palmitic acid, while 0.01 accounts for the 1% of naturally occurring ^{13}C in administered palmitic acid. Area under the curve of PDR was calculated using the trapezoidal rule and represents cumulative oxidation of dietary 1-^{13}C-palmitic acid.

5.3.3.4 Determination of Endogenous Hepatic Triglyceride Synthesis

On day 28 of each study phase, immediately following a fasted blood sample, subjects ingested 1.2 g/kg body water deuterium oxide (APE 99.9%) (Cambridge isotopes Inc., ACP Chemicals Inc. Montreal PQ). Body water was considered to be 70% total body weight. Deuterium oxide was micro-filtered prior to ingestion. On the morning of day 29, approximately 24 hrs after ingesting deuterium oxide, another fasted blood sample was taken. The exact time of deuterium ingestion and the day 29 blood sample was recorded.

Since VLDL particles are the richest source of newly synthesized fatty acids, VLDL-derived palmitic acid was used as an indicator for endogenous hepatic fatty acid synthesis (Leitch & Jones, 1993). VLDL from day 28 and 29 blood samples were separated from plasma using ultracentrifugation. Briefly, 1 ml of plasma was added to 3 ml saline (d=1.006 g/ml) in 4 ml polycarbonate ultracentrifuge tubes (Beckman-coulter, Mississauga, ON). Samples were centrifuged at 49 500 rpm for 4 hr. The VLDL was collected from the supernatant and heptadecanoic acid was added to VLDL as an internal standard. VLDL-derived lipids were extracted using chloroform: hexane (4:1). Samples were dried under nitrogen and derivitized via methylation using boron trifluroide (Sigma Aldrich, Oakville, ON), hexane and methanol (7:6:7 v/v/v) at 100°C for 55 min. Hexane and DD H_2O were added to samples which were then vortexed and centrifuged. The supernatant was collected, dried under nitrogen, resuspended in hexane and transferred to a GC vial for analysis.

Deuterium enrichment of methyl-palmitate was used to determine the fractional synthesis rate (FSR) of TG over a 24 hr period and was measured using gas chromatography (GC) (Agilent 6890) fitted with a SAC-5 column (Sigma Aldrich, Oakville ON) and coupled to a pyrolysis reactor and a Delta V Plus IRMS (Thermo Fisher Scientific Inc., Bremen, Germany). After separation by GC, fatty acids were pyrolized at 1450°C. The $\frac{^2H}{H}$ enrichment of methyl-palmitate was determined by the IRMS. Plasma water was used as the precursor pool from which ^2H_2O is acquired for fatty acid synthesis. Briefly, plasma water was isolated from whole plasma using centrifugal filtration (Millipore Ultrafree-0.5®, Fisher Scientific, Ottawa ON) at 12 000 g for 45 min. Plasma water was collected and $\frac{^2H}{H}$ enrichment was determined using a high temperature conversion/elemental analyzer coupled to a Delta V Plus IRMS. Methyl-palmitate and plasma water samples were analyzed in conjunction with a working reference gas consisting of a known $\frac{^2H}{H}$ enrichment. Samples were normalized against the working reference gas and reported as $\delta(‰)_{16:0}$ and $\delta(‰)_{H2O,}$ respectively. All IRMS data were analyzed using Isodat Software (Thermo Fisher Scientific Inc., Bremen, Germany**).** FSRs were calculated using the following formula.

$$FSR(day^{-1}) = \frac{\Delta\delta(‰)_{16:0}}{\Delta\delta(‰)_{\text{plasma H20}} \times 0.449} \times \frac{1440\ min}{\Delta time(\text{min})}$$

Where $\Delta\delta(‰)_{16:0}$ and $\Delta\delta(‰)_{\text{plasma H2O}}$ is the difference in palmitate and plasma water ^2H enrichment between day 28 and 29, respectively (Jones, 1996). To standardize all data over a 24 hr period, FSR data were multiplied by$\frac{1440\ min}{\Delta time(min)}$, where 1440 min is the number of minutes in 24 hrs and Δtime (min) is the difference in time between deuterium ingestion and the day 29 blood sample. Finally, 0.449 represents the correction factor for deuterium incorporation into endogenous palmitic acid and is based on previous research demonstrating that a maximum of 0.87 g ^3H g-atom/g-atom C is incorporated into newly synthesized fatty acids during adipose tissue lipogenesis (Wilke et al., 2009).

Net synthesis of VLDL-palmitic acid (g/d) were calculated using previously described methods, using the assumption that 300 mg VLDL-TG fatty acids/kg body weight are synthesized daily (Leitch & Jones, 1993).

5.3.4 Statistical Analysis

All statistical analyses were completed with SPSS version11.5 (SPSS Inc., Chicago, IL) using linear mixed model analysis of covariance (ANCOVA) with subject as a random factor. Repeated measures were used to determine the existence of effects of time and a time x treatment interaction. For thermogenic data and cumulative oxidation, total AUC as well as incremental AUC at each time point were compared between treatment groups. When appropriate, other factors were tested as covariates including, body weight, BMI, total lean mass, total fat mass, resting energy expenditure, and resting substrate oxidation. For thermogenic data, the interaction day × treatment was also tested as a covariate. In addition, the effect of treatment, gender, phase and treatment sequence were included in the model as fixed factors when their effect on the independent variable was significant. Comparisons between treatments were assessed using Bonferroni post hoc test. All data are presented as the mean±SEM. A level of significance at $p<0.05$ was used for all analysis.

5.4 RESULTS

5.4.1 Subject Dropout

Altogether 29 subjects were recruited for the study. Four subjects dropped out for personal reasons and two subjects left the study for medical reasons. Of the 23 volunteers that completed the entire 12 week trial, seven were men and 16 were women. Statistical analysis for all measurements revealed no effect of gender, age or an age by gender interaction.

5.4.2 Post Prandial Energy Expenditure, Thermic Effect of Food and Substrate Utilization

Results for post-prandial energy expenditure are summarized in **Figure 6**. Treatment had no effect on resting energy expenditure during week one (WF, 0.90±0.03 kcal/min; FPF, 0.90±0.03 kcal/min; WPF, 0.84±0.03 kcal/min) or on week four (WF, 0.89±0.03 kcal/min; FPF, 0.85±0.03 kcal/min; WPF, 0.93±0.03 kcal/min). For total energy expenditure, no difference between treatments was observed during week one (**Figure 6A)**. After four weeks, total energy expenditure was 5% lower (*p=0.007)* in the FPF group (333.0±3.6 kcal/330 min) compared to WF (349.3±3.6 kcal/330 min) **(Figure 6B)**. Analysis of incremental AUC indicate that post-prandial energy expenditure was reduced shortly following the breakfast meal. At 30 min after breakfast, FPF (29.0±0.2 kcal/30 min) and WPF (29.1±0.3 kcal/30 min) demonstrated lower (*FPF: p=0.004; WPF*: *p=0.025*) incremental energy expenditure compared to WF (29.8±0.3 kcal/30 min). After 90 min, FPF (64.9±0.8 kcal/60 min) tended (*p=0.054*) to maintain a reduced level of post-prandial energy expenditure compared to WF (67.5±0.9 kcal/60 min). Energy expenditure was positively correlated with calories consumed during the breakfast meal (*r=0.869, p<0.001*).

No differences in CHO oxidation were observed across treatments during week one **(Figure 7A)**. After four weeks, CHO oxidation tended to be lower (*p=0.075*) in the FPF group (44.7±2.1 g/330 min) compared to WF (51.2±0.1.9 g/330 min) (**Figure 7B**). Similar to total energy expenditure, week four CHO oxidation was positively correlated with calories during breakfast (*r=0.732, p<0.001).* No differences in total fat oxidation were observed across treatment groups during week one or week four (**Figure 8A-B)**.

Neither treatment demonstrated any effect on TEF during week one. However, TEF tended to be lower (*p=0.092*) during week four in the FPF group (51.0±3.5 kcal/330 min) compared to the WF group (62.3±3.2 kcal/330 min) (**Figure 9A**). When TEF data were normalized to energy consumed during the breakfast meal, a lower (*p=0.018)* proportion of calories were expended during diet induced thermogenesis with FPF (4.6±0.3%) compared to WF (5.7±0.3%) (**Figure 9B**). The proportion of calories expended during diet-induced thermogenesis with WPF (4.9±0.3%) did not differ (*p>0.*05) from other treatments.

5.4.3 Dietary Fat Oxidation

Percent dose recovery of $^{13}CO_2$ in breath and cumulative oxidation of 1-^{13}C palmitic acid is summarized in **Figure 10A-B**. Cumulative oxidation of dietary 1-^{13}C palmitic acid tended to be lower (*p=0.069*) in the FPF group (3.0±0.2%) compared to WF (3.4±0.2%). Analysis of incremental AUC data demonstrate that 370 min after consuming 1-^{13}C palmitic acid, a divergence in dietary fat oxidation was observed, with percent $^{13}CO_2$ recovered in the breath being higher (*p=0.*045) in the WPF group (0.96±0.05%) compared to FPF (0.81±0.05%) (**Figure 10Bi, 10Bii)**.

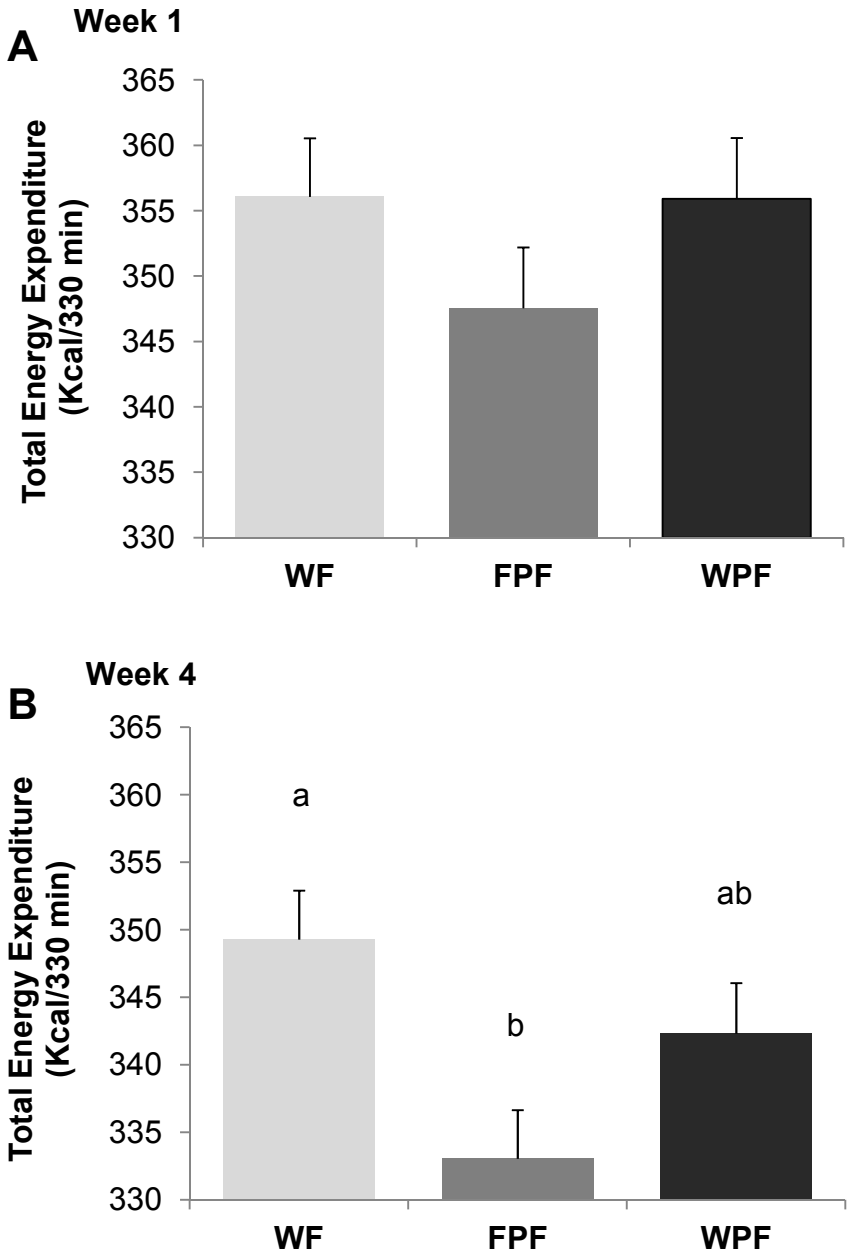

Figure 6 A-B. The effect of WF, FPF and WPF on total energy expenditure during **(A)** week one and **(B)** week four. Abbreviations: WF, White wheat flour; FPF, fractionated pea flour; WPF, whole pea flour. Endpoints with different letter subscripts are significantly different from each other (n =23).

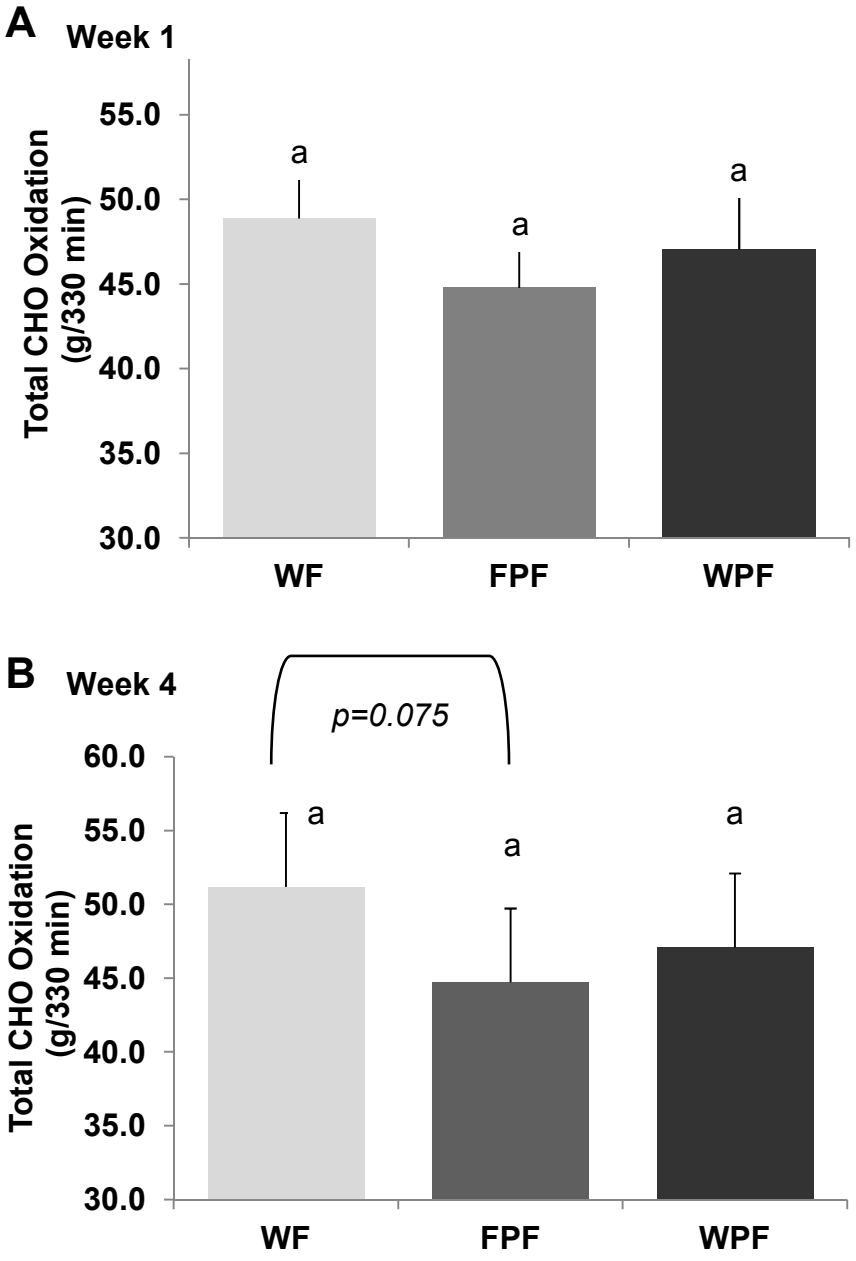

Figure 7 A-B. The effect of WF, FPF and WPF on total CHO oxidation during **(A)** week one and **(B)** week four. Abbreviations: WF, White wheat flour; FPF, fractionated pea flour; WPF, whole pea flour. Endpoints with different letter subscripts are significantly different from each other (n=23).

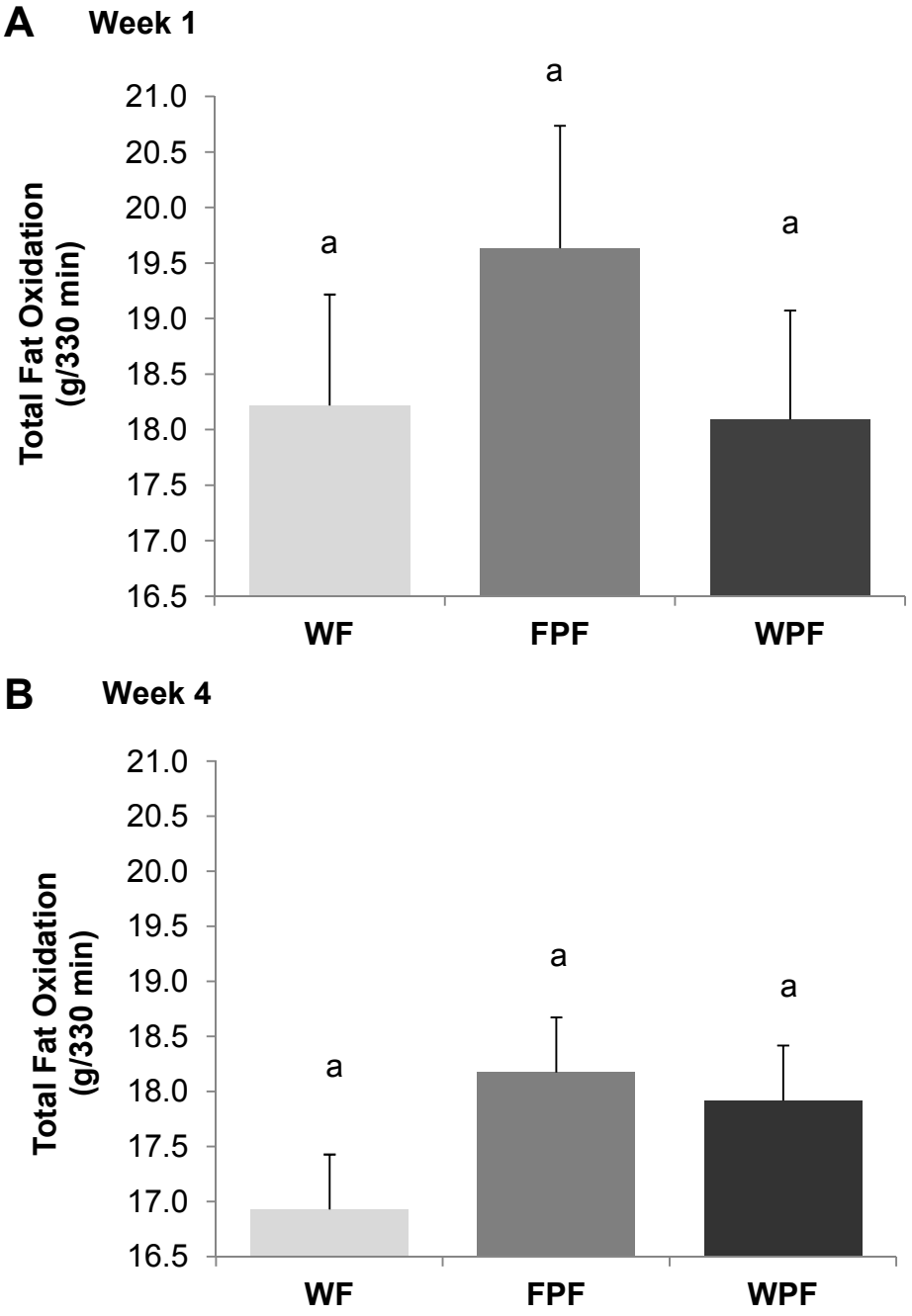

Figure 8 A-B. The effect of WF, FPF and WPF on total fat oxidation during **(A)** week one and **(B)** week four. Abbreviations: WF, White wheat flour; FPF, fractionated pea flour; WPF, whole pea flour. Endpoints with different letter subscripts are significantly ($p<0.05$) different from each other (n=23).

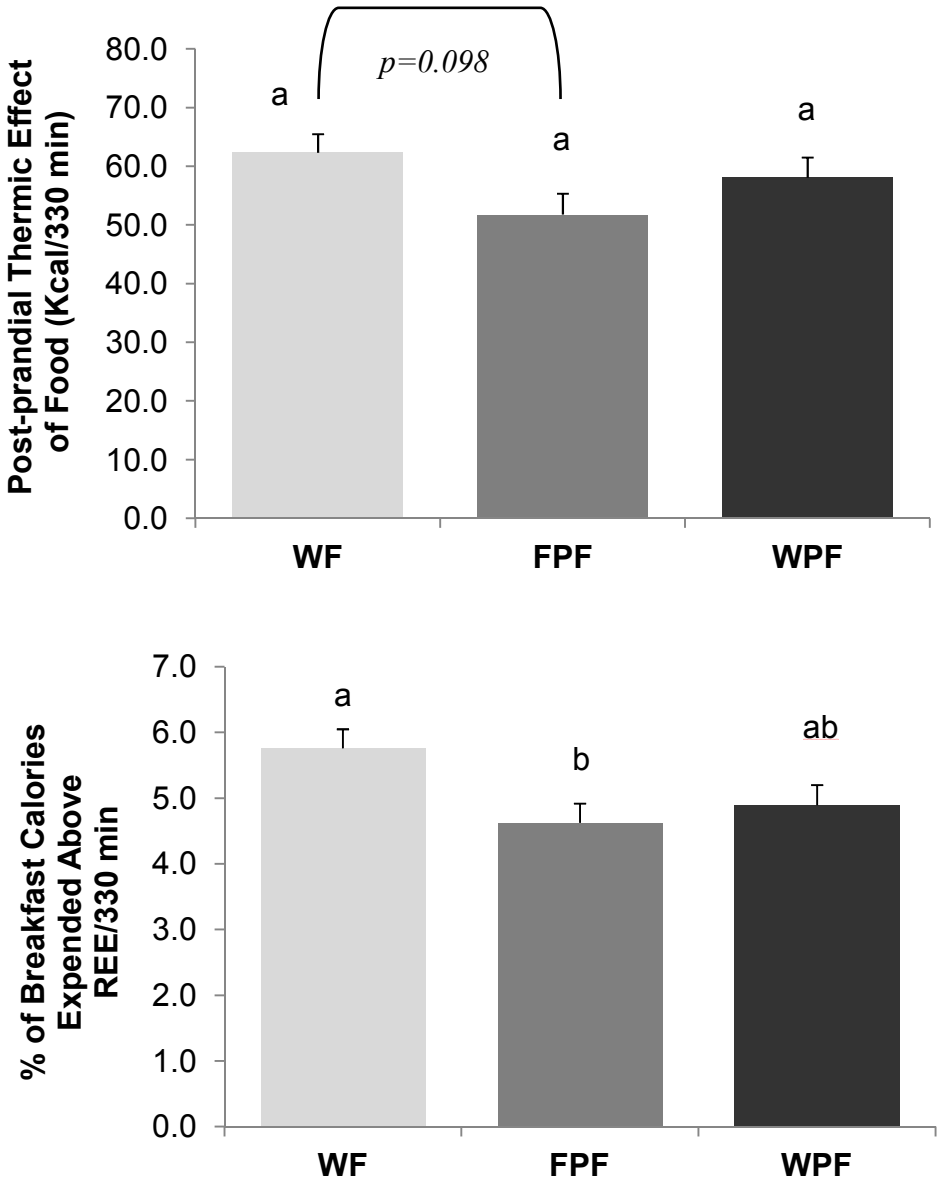

Figure 9 A-B. The effect of WF, FPF and WPF on **(A)** post-prandial thermic effect of food during week four and **(B)** post prandial thermic effect of food normalized to breakfast calories during week four. Abbreviations: WF, White wheat flour; FPF, fractionated pea flour; WPF, whole pea flour. Endpoints with different letter subscripts are significantly ($p<0.05$) different from each other (n=23).

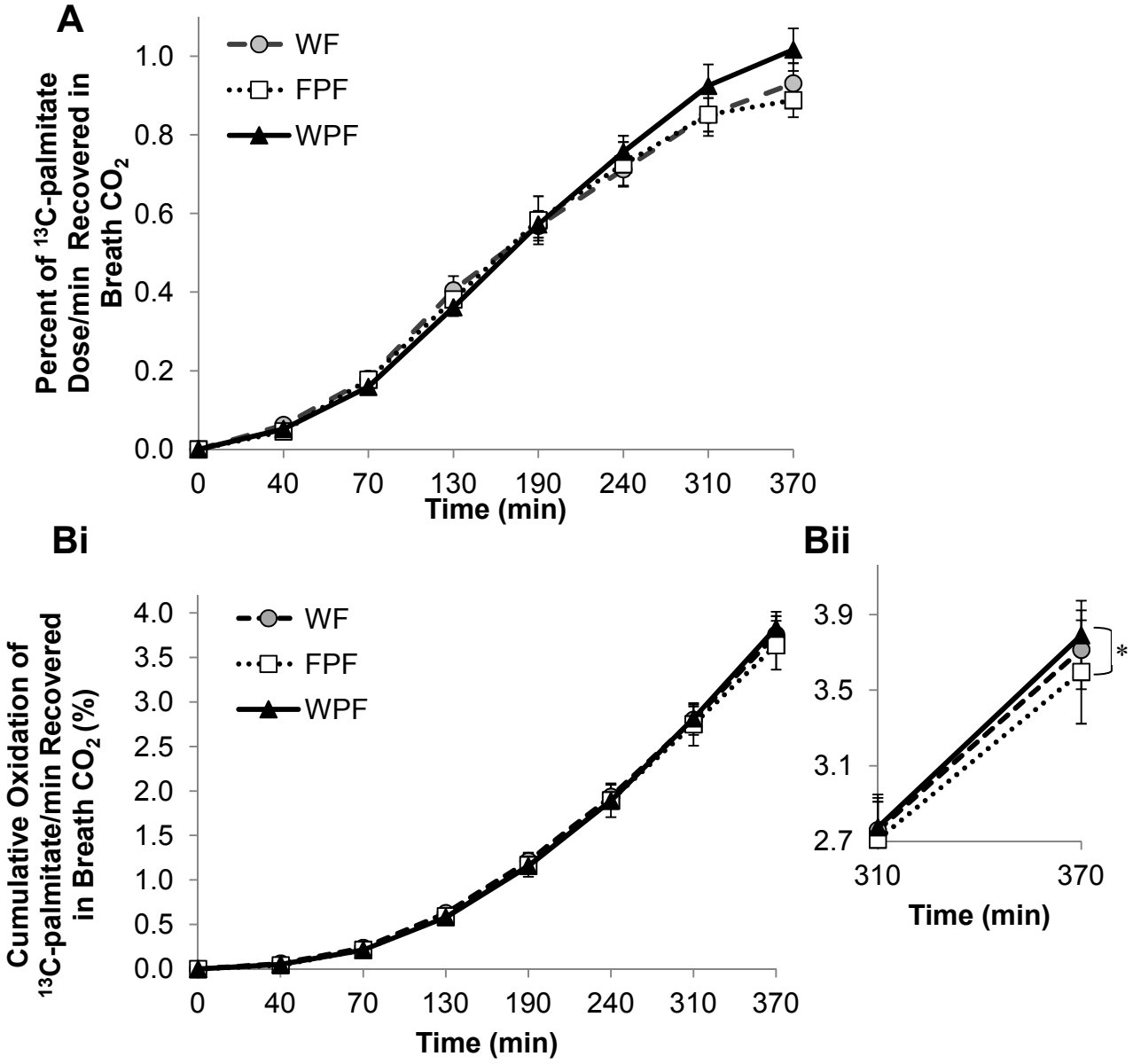

Figure 10 A, Bi, Bii. The effect of WF, FPF and WPF on the recovery of $^{13}CO_2$ recovered in breath secondary to oxidation of dietary ^{13}C-palmitic acid. **Figure 10A** represents the percent-dose recovery of $^{13}CO_2$ in breath. Figure 10 Bi represents cumulative oxidation of ^{13}C-palmitic acid/min recovered in breath CO_2. **Figure 10 Bii** represents an enhanced view of cumulative oxidation at 370 min. Abbreviations: WF, White wheat flour; FPF, fractionated pea flour; WPF, whole pea flour. * represents significant difference between treatments at $p<0.05$ (n=23).

5.4.4 Hepatic Fractional Triglyceride Synthesis

Deuterium incorporation into endogenous VLDL-derived palmitic acid was used as an index of TG synthesis. No differences in FSR for palmitic acid were found between WPF, FPF and WF (**Table 12**). Calculation of net TG synthesis indicated that the amount of VLDL-derived palmitic acid produced was also similar between treatments (**Table 12**)

Table 12. Fractional synthesis rates and net VLDL-palmitate synthesis for triglyceride synthesis between treatments

Treatment	FSR (day^{-1})	Net VLDL-Palmitate Synthesis (g/d)
WF	0.109 ± 0.0151	1.55 ± 0.201
FPF	0.128 ± 0.0151	1.66 ± 0.214
WPF	0.106 ± 0.0151	1.43 ± 0.141

Abbreviations: WF, white wheat flour; FPF, fractionated pea flour; FSR, fractional synthesis rate; WPF, whole pea flour

Mean ± SEM, n=23

5.5 DISCUSSION

The main finding of the present study was that regular consumption of fractionated yellow pea flour (hulls only) reduced post-prandial total energy expenditure, TEF, as well as CHO oxidation, compared to white wheat flour. In addition, the effects of FPF on total energy expenditure, post-prandial thermogenesis, and CHO oxidation were not demonstrated during week one, indicating that chronic intake of FPF is needed to elicit the observed changes in thermogenesis in the fed state.

Whole yellow pea flour is a source of insoluble fibre (**Table 10**). Although few studies have examined the effects of fibre on post-prandial thermogenesis, similar results were observed by Scalfi et al. (1987) whereby high fibre meals reduced post-prandial thermogenesis compared to a low fibre meal. The effect of fibre on post-prandial thermogenesis was attributed to the concomitant reduction in post-prandial glucose and insulin secretion (Scalfi et al., 1987). Delayed glucose absorption, alongside a decrease in insulin release would delay the exposure of glycolic pathways to glucose, thus reducing CHO oxidation and overall energy expenditure. In the present study, glucose levels were measured after each time increment and no differences in glucose response between treatment groups were observed (data not shown). Nonetheless, the lower energy expenditure shown in the present study with FPF is unlikely secondary to post-prandial glucose/insulin responses since the effects of FPF on energy expenditure only occurred after four weeks of treatment. Alternatively, these energetic responses may stem from mechanisms secondary to chronic ingestion of FPF. The need for chronic ingestion of high fibre meals to modulate energy expenditure is supported by Khossousi et al. (2008), whereby a single meal containing 15 g of fibre elicited no effect on energy expenditure after 6 hrs compared to the same meal containing 3 g of fibre. Data from the present study indicate that chronic ingestion of FPF is required to modulate energy expenditure.

Data demonstrate that the reduction in energy expenditure observed with FPF was due to lower CHO oxidation **(Figure 8).** Production of short-chain fatty acids (SCFA), specifically butyrate, as a result of microbial fermentation of fibre in the large intestine is a plausible mechanism to explain the FPF-related decrease in CHO oxidation. Butyrate has been shown to modulate hepatic CHO metabolism; indeed rats consuming glucose and butyrate demonstrated increases in post-prandial hepatic glycogen synthesis compared to rats fed glucose alone (Beauvieux et al., 2008). Enhanced glycogen synthesis would manifest itself as a reduction in CHO oxidation during indirect calorimetric measurements since glycogen synthesis pathways do not consume O_2 or produce CO_2. Furthermore, Anderson et al. (1984) demonstrated that butyrate reduced

the rate of glycolysis in rat hepatocytes, thereby reducing the entrance of CHO substrate into the Krebs cycle. In the same study, propanoate and valerate increased glycolysis, supporting the notion that different SCFA elicit various effects on CHO metabolism (Anderson & Bridges, 1984). Given that different sources of CHO give rise to different relative proportion of SCFA (Djouzi & Andrieux, 1997), the FPF utilized in the present study could have fostered the proliferation of butyrate producing bacteria in the large intestine. Compared to other sources of fibre, rats fed dietary pea fibre preferentially increased butyrate production relative to the total amount of SCFA produced (Stark & Madar, 1993). We hypothesize that the reduction in CHO oxidation was only observed in week four and not week one because of the time required for shifting intestinal microbial populations to those microflora that preferentially produce butyrate. Additional studies examining the effects of FPF on intestinal microbial populations are warranted.

Consumption of resistant starch has been shown to stimulate fat oxidation in conjunction with a moderate reduction in CHO oxidation (Higgins et al., 2004). The reason the FPF did not increase fat oxidation in this study cannot be determined with the present data. It has been established that, compared to lipid, CHO oxidation is relatively plastic in response to changes in diet composition and dietary substrate (Acheson et al., 1984; Flatt, 1995a). In addition, fat oxidation is higher in overweight and obese individuals (Flatt, 1995b). Thus, other biological factors, such as higher circulating fatty acids, may have concealed detectable compensatory responses secondary to effects of FPF or metabolites of FPF on CHO oxidation.

Whole pea flour failed to elicit the same effects on energy expenditure and CHO oxidation as FPF, albeit the two treatments were formulated to contain the same levels of pea-derived fibre. The whole pea flour used to produce the WPF treatment contains the cotyledon fraction of the pea as well as the majority of pea-derived proteins. Given that protein elicits the greatest effect on thermogenesis, WPF may have maintained energy expenditure levels compared to FPF (Johnston et al., 2002; Robinson et al., 1990).

Furthermore, previous literature examining the amino acid profile of de-hulled *Pisum sativum L*. indicates high levels of lysine and glutamate at 9.9 and 15.1 g amino acids/ 16 g N, respectively (Bhatty & Christison, 1984). Both lysine and glutamate have been shown to increase CHO oxidation and energy expenditure (Borel et al., 1998; Cornell et al., 1973; Iwashita et al., 2006; Iwashita et al., 2005). In addition, Iwashita et al. (2006) demonstrated that glutamate enhances CHO oxidation within 180 min of consuming the meal which mirrors results of the present study. The effect of glutamate on post-prandial thermogenesis could be secondary to the energetic requirements necessary for endogenous glucose production from dietary amino acids (Iwashita et al., 2006). Given that CHO oxidation is highly adaptive in the presence of excess CHO, enhanced gluconeogenesis from dietary amino acids could also foster an increase in the amount of endogenous CHO shuttled into oxidative pathways. Results from the present study suggest that the WPF treatment was able to maintain CHO oxidation, energy expenditure and diet induced thermogenesis, despite having the same levels of pea-derived fibre as FPF. Further investigation into the effects of the amino acid profile of yellow peas on energy expenditure and substrate oxidation is warranted to elucidate mechanisms of action.

Indirect calorimetry data from the present study showed higher fat oxidation in the FPF group, although the difference did not attain statistical significance. This finding contrasts with the breath $^{13}CO_2$ data, which demonstrated that fat oxidation tended to be lower in the FPF group compared to WPF and suggests that the majority of fat oxidized with FPF was from endogenous lipid. Dietary lipid is taken up by systemic tissues via the lymphatic system prior to reaching the liver. In addition, it is unlikely that fat oxidation in skeletal muscle would be enhanced by either treatment since study subjects were in the supine position during all indirect calorimetric and breath measurements, and muscle cells likely did not require higher than usual energy from fat. It is plausible that SCFA produced from FPF consumption modulated hepatic fat oxidation via mechanisms outlined above. Conversely, 270 min after consuming WPF, $^{13}CO_2$ in breath began to increase compared to FPF. Again, the amino acid profile of WPF could have induced fat

oxidation. In the same study outlined above by Iwashita et al. (2006), glutamate increased fat oxidation during indirect calorimetry, but was only evident after 210 min. The authors suggest that glutamate could stimulate fat oxidation directly by enhancing CHO oxidation during the early time period after the meal and force energy demands to be met by oxidizing more lipid during the later time period. In addition, the researchers speculated that glutamate can stimulate fat oxidation indirectly by increasing the flux of lipid through the Krebs cycle by increasing levels of α-ketogluarate and nicotinamide adenine dinucleotide redox potential (Bowtell & Bruce, 2002; Iwashita et al., 2006; Niihara et al., 1998). Using previously published literature (Bhatty & Christison, 1984) in conjunction with the dietary composition of the whole pea flour used in the present study, subjects received approximately 1.6 g/d pea-derived glutamate. If dietary glutamine from WPF affects fat oxidation, it is unlikely that any such effect is acute given the low dose of WPF-derived glutamine. However, rats supplemented with glutamine for 21 d demonstrated an accumulation in hepatic glutamine concentrations (Rogero et al., 2004) . Further research is needed to define the effects of chronic intake of whole and fractionated yellow peas on dietary fat oxidation.

Finally, neither treatment had any effect on hepatic TG FSR or net VLDL-palmitic acid synthesis. Moreover, results from net VLDL-palmitic synthesis correspond to previously published data (Leitch & Jones, 1993). The dietary factors most renowned for their effects on TG synthesis are marine-derived omega-3 fatty acids via the modulation of transcription factors such as peroxisome proliferator-activated receptor (Davidson, 2006). However, to our knowledge, no studies have concluded that pulse crops increase or decrease hepatic TG lipogenesis in humans. One would expect that because FPF modulated carbohydrate oxidation, TG synthesis would have been affected. However, studies indicate that glycogen storage is favored over CHO-derived TG synthesis. Moreover, CHO-derived TG synthesis only occurs with excess CHO ingestion and overfeeding for ≥ 2-3 d (Acheson et al., 1982; Acheson et al., 1988). In the present study, the amount of food each subject received was precisely controlled to prevent weight loss or weight gain. In addition, the present study employed a crossover-design,

thus, if a subject was overfed and created metabolic conditions that favor TG synthesis, this effect would have been demonstrated within energetic measurements in subsequent phases. We conclude that the addition of ½ cups of yellow peas/d to the diet does not modulate hepatic TG synthesis.

Results from the present study have clinical implications. First, these studies demonstrate that diets that foster higher CHO oxidation contribute to malnutrition in patients suffering from chronic obstructive pulmonary disease (COPD) because of the inability to increase respiration as a means of disposing of excess CO_2. Thus, diets and nutritional supplements that are higher in fat are often recommended to COPD patients to improve post-prandial respiratory function (Cai et al., 2003). The addition of fractionated pea flour to diets and nutritional supplements geared toward COPD could decrease post-prandial respiration via reduced CHO oxidation while maintaining CHO, fibre as well as overall food intake and thus, the risk of malnutrition in COPD patients. Second, the observation that chronic intake of WPF is able to maintain post-prandial energy expenditure compared to FPF could be important for overweight individuals implementing dietary lifestyle changes. Substituting WF with WPF would incorporate higher levels of fibre, antioxidants, and compared to WF thereby improving the overall quality of the diet with negligible effects on energy expenditure.

5.6 CONCLUSIONS

In summary, chronic dietary incorporation of FPF at a dose equivalent to ½ cup whole yellow peas/d decreased total energy expenditure, TEF, and carbohydrate oxidation compared to WF. Conversely, chronic intake of WPF was able to negate the effects of pea-derived fibre on post-prandial energetics. Although data from the present study suggest that WPF could increase the oxidation of exogenous lipid, studies that investigate the affect of WPF on post-prandial dietary fat oxidation beyond 6 hrs are needed to confirm these results. The present study demonstrates that chronic ingestion of different fractions of yellow peas imposes distinctive effects on post-prandial energy expenditure and substrate utilization and justifies further exploration of the effects of other pulse crops on post-prandial energetics.

5.7 ACKNOWLEDGMENTS

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BRIDGE TO CHAPTER VI

Using a diet and energy controlled paradigm, Chapters III, IV and V have demonstrated that whole and fractionated yellow peas modulate circulating insulin levels, estimates of insulin resistance, regional adipose deposition, post-prandial glucose response, post-prandial energy expenditure and substrate utilization in humans. However, other clinical endpoints such as body composition and circulating lipid levels were unaffected by pulse-crop-derived treatments. Given that whole and fractionated yellow peas vary in nutrient composition, the USDA recommended dosages of whole and fractionated yellow pea flour utilized in Chapters III & V could have been insufficient in specific bioactives required to modulate certain clinical endpoints. For example, pulse crops, including yellow peas, are excellent sources of fibre. While fibre is believed to decrease circulating lipid levels by facilitating lipid excretion in the intestine and/or modulate hepatic lipid synthesis, the relative dosages of yellow pea fibre used in Chapters III could have been insufficient to impose a lipid-lowering effect given the large amount of food that subjects consumed to prevent fluctuations in weight. Thus, studies investigating the effects of higher doses of whole and fractionated pulse crops on clinical endpoints should assist in delineating the effects of pea-based flours on clinical endpoints that were unresponsive to quantities of pulses consumed in Chapters III & V.

Given the growing interest in the symbiotic relationship between gastrointestinal microflora and the host, dietary interventions that promote the proliferation of "good" colonic bacteria are gaining widespread attention. Prebiotics are dietary components that resist digestion in the small intestine and promote the growth of beneficial colonic bacteria. Fibres are the most predominant undigested dietary constituents that reach the colon, and despite the high levels of fibre found in pulse crops, their prebiotic effects remain undetermined. Furthermore, given that protein can also modulate the gastrointestinal microbiome, whole yellow peas could impose distinct prebiotic effects not observed with consumption of fractionated yellow peas. The discovery that whole and/or fractionated yellow peas facilitate prebiotic effects will support further

investigation into the health benefits of pulse crops as functional food ingredients.

Using Golden Syrian hamsters as an appropriate animal model to investigate the effects of dietary interventions on lipid metabolism, the first objective of the following study was to determine if high doses of whole and fractionated yellow pea flours impose distinct effects on circulating cholesterol, triglyceride, insulin and glucose levels as well as body composition and energy expenditure. The second objective was to conduct a pilot study to determine if whole and fractionated yellow peas facilitate perturbations in the caecal microbiome.

CHAPTER VI

WHOLE AND FRACTIONATED YELLOW PEA FLOURS MODULATE INSULIN, GLUCOSE, ENERGY EXPENDITURE AND THE GASTROINTESTINAL MICROBIOME IN GOLDEN SYRIAN HAMSTERS

Christopher P.F. Marinangeli[1], Dennis Krause[1, 2], Scott V. Harding[1], Todd C. Rideout[1], Fuqin Zhu[2], and Peter J.H. Jones[1]

[1] University of Manitoba, Richardson Centre for Functional Foods and Nutraceuticals, 196 Innovation Drive, Winnipeg, Manitoba, R3T 2N2, Canada

[2] University of Manitoba, Department of Animal Science, Animal Science Building Winnipeg, Manitoba, R3T 2N2, Canada

To whom correspondence should be directed:
Dr. Peter J.H. Jones, PhD
Richardson Centre for Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive
Winnipeg, MB, CANADA
R3T 2N2
Tel: +1 204 474 8883
Fax: +1 204 474 7552
Email: peter_jones@umanitoba.ca

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Running Title: Metabolic actions of yellow pea flour

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6.1 ABSTRACT

The objective was to evaluate the effects of whole and fractionated yellow peas on circulating lipids, glucose and insulin levels, energy expenditure and body composition, as well as to assess their prebiotic actions in Golden Syrian hamsters. Forty five hamsters consumed a hypercholesterolemic diet for 28 d, then were randomly assigned to one of three treatment groups: control (CON), whole pea flour (WPF) and fractionated pea flour (hulls only) (FPF). WPF and FPF were incorporated into diets at 10% at the expense of cornstarch. WPF and FPF feeding produced negligible effects on circulating cholesterol and triglyceride levels. However, both WPF (56.76 ± 9.22 pmol/ml, $p= 0.002$) and FPF (89.27 ± 19.82 pmol/ml, $p=0.032$) reduced circulating insulin levels compared to the CON group (131.70 ± 17.70 pmol/ml). Moreover, FPF decreased ($p=0.03$) circulating glucose levels (6.26 ± 0.51 mmol/l) compared to CON (8.27 ± 0.81 mmol/L). Energy expenditure analysis revealed that hamsters consuming WPF demonstrated a higher ($p=0.036$) oxygen consumption (2.00 ± 0.31 ml O_2/g lean body mass) versus the CON group (1.56 ± 0.089 ml O_2/g lean body mass). Analysis of caecal digesta showed that WPF produced shifts in the abundance of microbial taxa with the most predominant changes occurring within the phylum Firmicutes. In conclusion, yellow peas and their constituents should be investigated as future functional food ingredients that help prevent and manage disease.

6.2 INTRODUCTION

Pulse crops, including lentils, chickpeas, beans and peas, are routinely endorsed as components of a healthy diet (Leterme, 2002). Given that pulses are characterized as excellent sources of fibre, protein and antioxidants, as well as non-specific nutritional compounds, numerous clinical trials have attempted to delineate the health benefits of these crops. Nonetheless, results regarding the effects of pulse crops on circulating lipid (Jenkins et al., 1983b), glucose and insulin levels (Nestel et al., 2004) as well as body weight and body composition (Yang et al., 2007), have been inconsistent. In addition, pulse-derived bioactive components that coincide with specific clinical endpoints have not been identified. For example, it is unknown whether reductions in body weight and fat deposition with consumption of pulses are secondary to pulse-crop induced satiety via pulse-derived fibre (Haber et al., 1977) , impaired nutrient absorption secondary to anti-nutritional factors such as trypsin inhibitors (Wang & McIntosh, 1996), a direct effect of nutritional components acting on biological pathways that modulate fat and lean mass compartments (Yang et al., 2007), or a combination of factors.

Currently, the USDA recommends that individuals consume ½ cup of pulses/d as part of a healthy diet (USDA.gov, 2009). In fact, many studies conducted in humans utilize ≈ ½ cup pulses, cooked or as flour each day as a standard treatment dose for investigating their health benefits. However, given the variability of pulse crops on the aforementioned health outcomes, one can question whether the dose of pulse crops utilized is, in some cases, too low to elicit an effect, especially in studies where dietary intakes are not controlled. For example, fibres are often credited as the efficacious lipid-lowering component of pulse crops via their ability to facilitate lipid excretion in the feces by binding dietary fat and bile acids in the intestine (Dubois et al., 1993; Ebihara & Schneeman, 1989; Elhardallou, 1992; Jonnalagadda et al., 1993; Kahlon et al., 2004; Kingman et al., 1993). Nevertheless, in situations of high fat intake where subjects are permitted to consume their habitual diet, we suggest that the amount of pulse-derived

fibre in the recommended ½ cups/d whole pulses could be insufficient to bind adequate dietary lipid, decrease lipid absorption and reduce circulating cholesterol and/or triglyceride levels. One way to improve the effectiveness of whole pulse crops as functional foods is to utilize post-harvesting processes that separate and concentrate bioactive constituents. Pulse crops such as peas are easily fractionated into two components, including the outer region known as the hull and the inner region known as the cotyledon. Our nutrient analysis demonstrates that whole yellow peas, complete with hull and cotyledon, are ≈20% fibre, ≈20% protein and ≈50% available carbohydrate (Medallion Labs, Minneapolis, Minnesota, USA). However, when analyzed independent of the cotyledon, the hull is ≈85% fibre, indicating considerable variability in the macronutrient content between fractions. Utilization of specific fractions as functional ingredients could provide adequate dosages of bioactive constituents that modulate clinical endpoints.

Finally, prebiotics are undigested dietary components that modulate the gastrointestinal microbiome (Gibson & Roberfroid, 1995). Consuming nutrients that bypass digestion in the small intestine can facilitate growth of beneficial colonic microflora, improving local and systematic immunity, inflammation, and metabolism (Gibson & Roberfroid, 1995; Lomax & Calder, 2009a; Looijer-van Langen & Dieleman, 2009; Nishimura et al., 2010; Ryz et al., 2009). Studies demonstrate that diets varying in fibre (Lomax & Calder, 2009a), protein (De Palma et al., 2009) and fat (Paturi et al., 2010) differentially affect colonic microbial populations. The prebiotic nature of whole and fractionated pulse crops has yet to be investigated and given the distinct nutrient composition of whole and fractionated yellow peas, one can hypothesize that components of the pea cotyledon and hull elicit discrete prebiotic effects.

Therefore, our first objective was to investigate the effects of high doses of whole and fractionated (hulls only) yellow peas on circulating lipid, glucose and insulin levels, as well as body composition and body weight in Golden Syrian Hamsters. The Syrian

hamster has been identified as an ideal animal model to investigate effects of dietary interventions on cholesterol levels given that its lipid metabolism resembles that of humans (Briand et al., 2010; Kris-Etherton & Dietschy, 1997). A second objective was to conduct a pilot study to determine if components of yellow peas exert identifiable prebiotic effects on caecal microbial populations.

6.3 MATERIALS AND METHODS

6.3.1 Animals and Diets

Forty five male Golden Syrian hamsters (*Mesocricetus auratus*) at two weeks of age were utilized for the study. Hamsters underwent a two-week acclimatization period where they consumed regular rodent chow ad libitum with free access to water. After acclimatization, animals were fed a hypercholesterolemic AIN93-G diet containing 0.25% cholesterol for 28 d (**Table 13**) (Kassis et al., 2007; Yang et al., 2006) and were assigned to one of three treatment groups: control (CON), whole pea flour (WFP), or fractionated pea flour (FPF). The WPF and FPF treatments were added to diets at 10%, in place of cornstarch (**Table 13).** The energy density of each treatment diet is outlined in **Table 13.** Both WFP and FPF treatments were acquired from Best Cooking Pulses Inc. (Portage La Prairie, MB, Canada). Prior to milling, yellow peas were dried on the vine and subsequently harvested. Whole yellow peas were milled without further processing. In order to produce FPF, hulls were separated from their cotyledons and subsequently milled into flour. The nutritional composition of WPF and FPF is summarized in **Table 14.** The study protocol received ethical approval from the University of Manitoba Animal Care Committee.

Table 13. Composition of hypercholesterolemic diets consumed by hamsters

Constituents (wt.%)	Treatment Groups		
	CON	**WPF**	**FPF**
Whole pea flour	-	10.0	-
Fractionated pea flour	-	-	10.0
Casein	20.0	20.0	20.0
Corn starch	26.0	16.0	16.0
Sucrose	33.03	33.03	33.03
Lard/Sunflower mix	5.0	5.0	5.0
Cellulose	10.5	10.5	10.5
DL-methionine	0.5	0.5	0.5
Mineral mixture	3.5	3.5	3.5
Vitamin mixture	1.0	1.0	1.0
Choline bitartrate	0.2	0.2	0.2
BTH	0.02	0.02	0.02
Cholesterol	0.25	0.25	0.25
Energy (KJ/100g diet)	1528	1486	1392

Abbreviations: CON, control; WPF, whole pea flour; FPF, fractionated pea flour, BTH, butylated hydroxytoluene

[1] Vitamin mix AIN-76A (CA40077; Harlan Teklad, Madison, WI, USA) ("Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies.," 1977).

[2] Mineral mix AIN-93M (TD94047, modified for hamsters; Harlan Teklad, Madison, WI, USA) (Reeves et al., 1993).

Table 14. Nutritional composition of the WPF and FPF treatments

Treatment Flour	WPF[1]	FPF[1]
Energy kJ/100 g	1 281	343
Total carbohydrate (%)	66.3	89.0
Available carbohydrate (%)	50.7	12.7
Fibre (%)[§]	17.6	84.2
Soluble (%)	2.0	7.9
Insoluble (%)	15.6	76.3
Total Fat (%)	1.84	0.63
Polyunsaturated fat (%)	0.98	0.31
Monounsaturated fat (%)	0.44	0.13
Saturated (%)	0.34	0.16
Protein (%)	21.6	6.39

Abbreviations: WPF, whole pea flour; FPF, fractionated pea flour

[1] Analysis conducted by Medallion Labs (Minneapolis, MN, USA).

[§] Analysis of total, soluble and insoluble fibre was completed by Medallion Labs using AOAC method 991.43.

6.3.2 Study Protocol

Hamsters were systematically randomized into study groups to ensure an even distribution of starting body weights across treatments. Body weights were measured weekly, while food consumption was assessed every 3 d by subtracting spillage from the amount of food remaining in feeders. On day 20, energy expenditure was assessed using a respiratory gas exchange system for rodents (MM-100 CWE, Inc., Pennsylvania, PA, USA). Animals were placed in individual metabolic chambers and oxygen consumption was measured at one minute intervals over a two hour period. On day 27, a 24 hr fecal collection was initiated. Feces were stored at -80°C until analysis.

On day 28, hamsters were anaesthetized with inhaled isoflurane and blood was collected by cardiac puncture. Animals were subsequently euthanized by overdose of sodium pentobarbital. Following euthanization, body composition was assessed using dual energy x-ray absorptiometry (DEXA) (Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA), alongside Encore 2005 software version 9.30.044 (GE Healthcare, Madison, WI, USA). Following DEXA, necropsy of the hamsters' caecums and their contents were performed. Caecums were frozen immediately in liquid nitrogen and stored at -80°C until analysis. Blood was centrifuged at 3000 rpm at 4°C to separate serum and plasma from red blood cells, and subsequently stored at -80°C until analyzed. Food was removed from cages 10 hrs prior to sacrifice.

6.3.2.1 Blood Lipid, Glucose and Insulin Analysis

Fasting total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglyceride (TG) and glucose levels were determined enzymatically using a clinical chemistry analyzer (Vitros 350, Ortho-clinical Diagnostics Inc. Rochester, NY, USA). Non-HDL-C was calculated as the difference between measured TC and HDL-C, and included VLDL cholesterol (VLDL-C), LDL-cholesterol (LDL-C) and intermediate density lipoprotein-cholesterol (IDL-C). As demonstrated previously (Li et al., 2009), circulating insulin levels were determined using ELISA (Millipore, Linco Research, MO, USA).

6.3.3 Prebiotic Analysis of Fecal Samples

6.3.3.1 Fecal DNA Extraction

DNA from caecal contents was extracted using ZR Fecal DNA Kit (D6010, Zymo Research Corp., Orange, CA). The bead beating step utilized to mechanically lyse cells was extend to 3 min. Quality of DNA was evaluated using gel electrophoresis. Extracted DNA from hamsters within each treatment group was subsequently pooled together for PCR amplification and terminal restriction fragment length polymorphism (TRFL-P) analysis.

6.3.3.2 PCR Amplification and Terminal Restriction Length Polymorphism Analysis

PCR amplification of DNA samples was conducted using previously described methodologies (Khafipour et al., 2009; Sepehri et al., 2007). Briefly, V1 and V2 regions of ribosomal DNA (rDNA) genes were amplified using universal bacterial primers specific for prokaryotic sequences, 27f (5'-AAGAGTTTGATCATGGCTCAG-3') and 342r (5'-TGCTGCCTCCCGTAG-3'). To allow for detection during electrophoresis, the forward primer was fluorescently labeled (WellRED D4dye, Sigma-Proligo, St. Louis, MO, USA) (Khafipour et al., 2009). The PCR amplification program was as follows, one cycle at 94°C for five min; then 36 cycles at 94°C for one min; 56°C for one min; 72°C for two min; and a final extension at 72°C for five min. Terminal restriction fragments were generated by digesting PCR products with *Hha*I (15 µL of PCR product, 10 units of *Hha*I, 1X *Hha*I buffer, and 20 µg of bovine serum, New England Biolabs, Ipswich, MA, USA) at 37°C for three hours. Fragment lengths were determined using a CEQ 8800 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA, USA). Two µl of fluorescently labeled PCR product alongside 29.5 µL of sample loading solution, and one µL of 600 basepair (bp) DNA standard (Beckman Coulter, Inc., Fullerton, CA, USA)

were mixed for analysis. An electropherogram with variable peak sizes was generated for each within treatment pooled DNA sample. Peaks of different sizes corresponded to disparate operational taxonomic units (OTU).

6.3.3.3 Terminal Restriction Fragment Length Polymorphism Analysis

Fragments were analyzed using CEQ software (Version 9.0; Beckman Coulter Inc., Fullerton, CA) using a two bp binning parameter. Only fragments that constituted > 1% total abundance were included in the analysis. Data from the OTU profiles were grouped based on treatment and used numerical analysis. Data are presented as percent abundance of microbial populations within phyla and further segregated into the taxa, class and order.

6.3.4 Statistical Analysis

All statistical analyses were completed using SPSS software version 11.5 (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was used to detect differences between study groups using treatment, block and the interaction between block and treatment as fixed factors. Covariance parameters were included in the statistical analysis (ANCOVA) when appropriate. Body weight gain and energy intake were used as covariates for statistical analysis of body fat mass. Normality was assessed using the Shapiro-Wilks test as well as evaluating linearity and clustering in normal and disturbed Q-Q plots, respectively. Reciprocals of data corresponding to glucose, total oxygen consumption and oxygen consumption per gram lean body mass were used to achieve homogeneity for statistical analysis while insulin data was log-transformed. All transformed data was back-transformed for reporting. Data analyzed by ANOVA and ANCOVA utilized Tukey's post-hoc analysis and Bonferroni's correction to determine which groups differed from each other, respectively. For analysis of caecal digesta, groups of microflora that demonstrated at least a three-fold change in microbial

abundance between treatments were subjected a chi square test of independence using a 2 x 2 contingency table and Fisher's Exact Test. For all statistical analysis, significance was set at $p < 0.05$ and trends were identified at $p < 0.1$.

6.4 RESULTS

6.4.1 Animal Growth and Feed Intake

Growth rates, food intake and energy intake of animals are summarized in **Table 15**. Data demonstrated no significant differences between treatment groups in percent body weight gain and food intake between day 1 and day 28 of the study. However, animals consuming FPF consumed 8% less (*p=0.*028) energy/d compared to CON. Energy intakes between FPF and WPF were not different from each other.

Table 15. Differences in body weight gain, average food intake and energy intake between treatment groups

	Treatment			p value
	CON	**WPF**	**FPF**	
Body weight gain (%)	7.0 ± 2.1	2.4 ± 2.1	3.5 ± 2.1	0.285
Average feed intake (g/d)	9.7 ± 1.1	9.5 ± 0.7	9.8 ± 1.1	0.664
Energy intake (KJ/d)	148.6 ± 4.4^{a}	141.3 ± 2.7^{ab}	$136.1 \pm 3.8^{b\S}$	0.033

Abbreviations: CON, control; WPF, whole pea flour; FPF, fractionated pea flour

Mean ± SEM

Values with different letter subscripts are significantly different from each other

§ $p<0.05$

6.4.2 Lipids, Glucose, Insulin, Body Composition and Oxygen Consumption

Neither treatment had any effect on TC, HDL-C, non-HDL-C and TG levels. FPF reduced (*p=0.03*) circulating glucose levels 24% compared to CON (**Table 16**). WPF and FPF decreased insulin 60% (*p= 0.002*) and 32% (*p=0.032*), respectively, compared to the CON group. While DEXA analysis revealed that WPF and FPF had no effect on total mass and lean body mass, total fat mass tended to be lower in the WPF (*p=0.079*) and FPF (*p=0.090*) groups compared to CON (**Table 16**). However, this trend disappeared when statistical analysis controlled for body weight gain and energy intake. Finally, no significant changes (*p=0.322*) in total oxygen consumption were observed between all three treatment groups (**Table 16**). Nonetheless, when normalized to total lean body mass, oxygen consumption with WPF significantly increased (*p=0.036*) 28% above animals receiving the CON treatment (**Table 16**). Compared to WPF and CON, FPF did not modulate energy expenditure.

6.4.3 Prebiotic Effects of Whole and Fractionated Pea Flours

Table 17 demonstrates that FPF and WPF induced variable effects on the abundance of certain caecal microbial populations of hamsters. Firmicutes represented the most abundant bacterial phylum and demonstrated the most profound shifts in microbial populations **(Table 17 & Figure 11)**. Although substantial changes were not demonstrated in total Clostridia, the abundance of unclassified Clostridia were 80 and 90% lower ($p<0.001$) in FPF and CON relative to the WPF group, respectively. In addition, compared to WPF, the abundances of Bacilli were reduced ($p=0.029$) 85% in the FPF group which were mostly secondary to a trend (*p=0.06*) in higher levels of Lactobacillales in WPF compared to FPF. Fisher's Exact Test revealed no other significant changes within microbial groups between treatments that demonstrated a ≥ three-fold difference in microbial abundance (**Table 17 & Figure 11**). Nonetheless, hamsters receiving WPF expressed a three to four fold increase in Bacilli and

Lactobacillales, respectively compared to CON **(Figure 11)**. Although the phylum Protobacterium represents the second most abundant group of bacteria, shifts in microbial populations between treatments were less substantial compared to those noted within the Firmicutes, with the exception of Gammaproteobacteria. The relative increase in Gammaproteobacteria in WPF was secondary to a two and three-fold decrease in the abundance of Enterobacteriales within the CON and FPF groups, respectively **(Table 17)**.

Table 16. The effect of treatments on lipids, glucose, insulin, body composition and energy expenditure

Clinical Endpoint	Treatments			p value
	CON	WPF	FPF	
Lipids				
Total cholesterol (mmol/L)	7.79 ± 0.46	7.85± 0.22	8.20 ± 0.25	0.621
HDL-C (mmol/L)	3.75 ± 0.09	3.82 ± 0.03	3.84 ± 0.04	0.582
Non HDL-C (mmol/L)	3.79 ± 0.09	4.02 ± 0.03	4.36 ± 0.04	0.322
Triglycerides (mmol/L)	4.16 ± 0.35	4.59 ± 0.21	4.55 ± 0.22	0.664
Glucose (mmol/L)	8.27 ± 0.81^{a}	6.75 ± 0.39^{ab}	6.26 ± 0.51^{b§}	0.040
Insulin (pmol/mL)	131.70 ± 17.70^{a}	56.76 ± 9.22^{b§}	89.27 ± 19.82^{b§}	0.010
Body composition				
Total mass (g)	127.33 ± 0.0038	121.33 ± 0.0030	120.67 ± 0.0031	0.166
Total fat mass (g)	64.67 ± 2.50^{a}	57.80 ± 3.12^{b¶}	58.00 ± 2.49^{b¶}	0.051
Total lean mass (g)	58.40 ± 2.12	59.20 ± 0.96	59.47 ± 1.57	0.862
Bone mineral content (g)	3.06 ± 0.074	3.04 ± 0.087	3.07 ± 0.071	0.963
Energy expenditure				
Total oxygen consumption (ml)	90.37 ± 5.63^{a}	116.50 ± 16.97^{b¶}	107.94 ± 14.79^{ab}	0.322
Oxygen consumption (ml/g lean bw)	1.56 ± 0.089^{a}	2.00 ± 0.31^{b§}	1.85 ± 0.27^{ab}	0.040

Abbreviations: CON, control; WPF, whole pea flour; FPF, fractionated pea flour; bw, body weight

Mean ± SEM

Values with different letter subscripts are significantly different from each other

¶ $p < 0.1$

§ $p < 0.05$

Table 17. Percent abundance of caecal microbial populations analyzed by TRFL-P

Taxonomic Classification	Treatments		
	CON	WPF	FPF
	% Abundance		
Phylum Lentisphaerae	**0.0 ± 0.3**	**0.7 ± 0.3**	**0.8 ± 0.3**
Phylum Bacteroidetes	**2.8 ± 0.8**	**1.5 ± 0.8**	**0.0 ± 0.8**
Phylum Firmicutes	**91.5 ± 1.2**	**89.6 ± 1.2**	**93.6 ± 1.2**
Class Bacilli	1.4 ± 0.4^{ab}	$5.2 \pm 1.4^{a¥}$	0.8 ± 1.4^{b}
Order Lactobacillales	1.4 ± 1.1^{ab}	$4.4 \pm 1.1^{b¶}$	0.8 ± 1.1^{a}
Order Bacillales	0.0 ± 0.2	0.7 ± 0.2	0.0 ± 0.2
Class Clostridia	87.3 ± 2.4	81.5 ± 2.4	89.4 ± 2.4
Order Clostridiales	84.5 ± 7.8	61.5 ± 7.8	85.4 ± 7.8
Unclassified Clostridia	2.8 ± 5.5^{a}	$20.0 \pm 5.5^{b§}$	4.1 ± 5.5^{a}
Unclassified Firmicutes	2.8 ± 0.1	3.0 ± 0.1	3.3 ± 0.1
Phylum Proteobacteria	**5.6 ± 0.8**	**8.1 ± 0.8**	**5.7 ± 0.8**
Class Alphaproteobacteria	1.4 ± 0.4	0.7 ± 0.4	0.0 ± 0.4
Class Epsilonproteobacteria	2.8 ± 0.4	1.5 ± 0.4	1.6 ± 0.4
Class Gammaproteobacteria	1.4 ± 0.7	3.7 ± 0.7	1.6 ± 0.7
Order Pasteurellales	1.4 ± 0.2	0.7 ± 0.2	0.8 ± 0.2
Order Enterobacteriales	0.0 ± 0.9	3.0 ± 0.9	0.8 ± 0.9
Class Betaproteobacteria	0.0 ± 0.7	2.2 ± 0.7	1.6 ± 0.7
Unclassified Proteobacteria	0.0 ± 0.3	0.0 ± 0.3	0.8 ± 0.3

Abbreviations: CON; control; WPF, whole pea flour; FPF, fractionated pea flour

Mean±SEM

Values with different letter subscripts are significantly different from each other

Significance was set at $p<0.05$

¶ $p<0.1$

¥ $p<0.05$

§ $p<0.001$

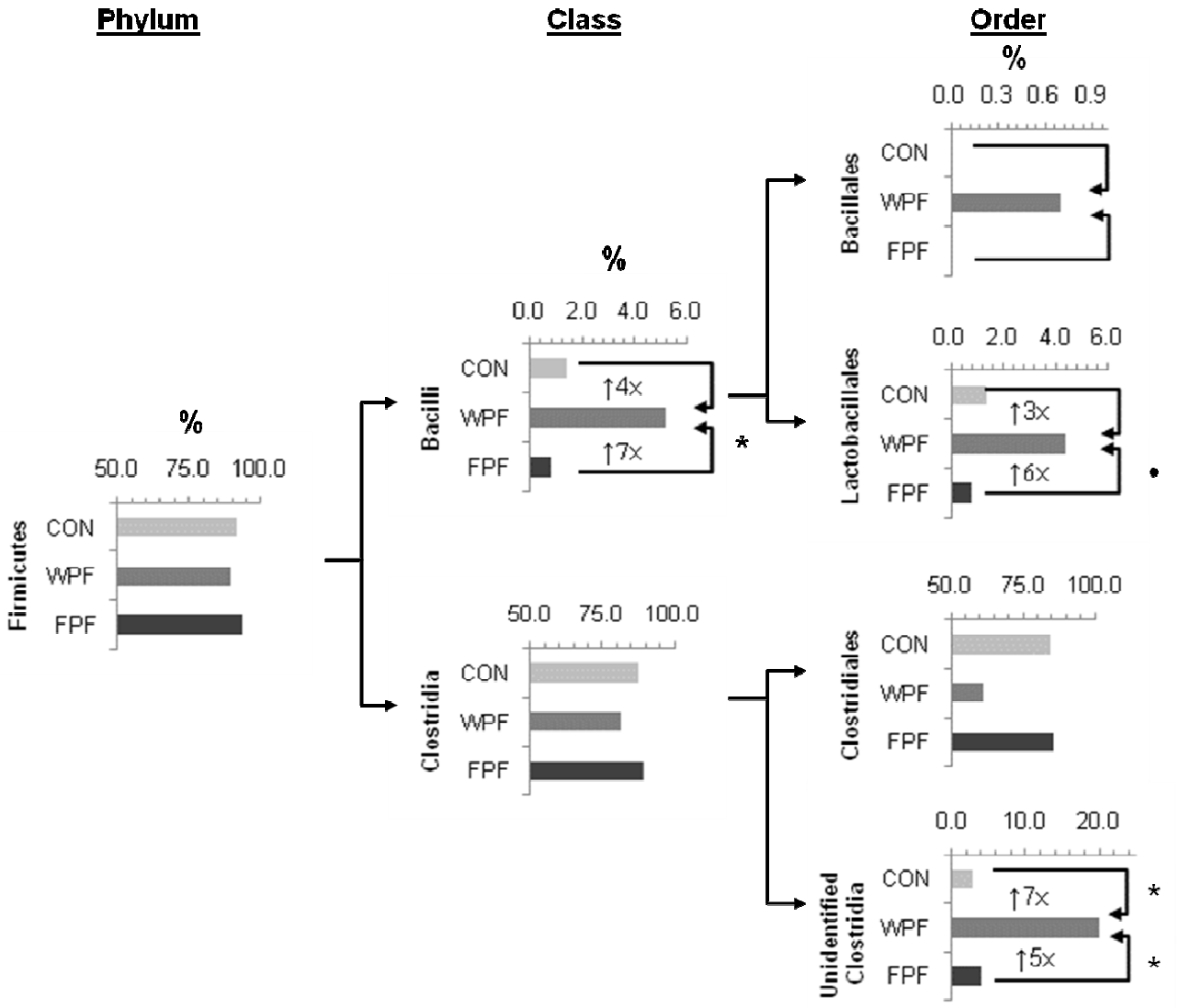

Figure 11. TRFL-P analysis of caecal digesta demonstrating microbial shifts in percent abundance within the phylum Firmicutes. Abbreviations: CON, control; WPF, whole pea flour; FPF, fractionated pea flour. * denotes $p<0.05$ and • denotes $p<0.1$.

6.5 DISCUSSION

The present study demonstrates that, although whole and fractionated yellow peas imposed similar reductions in circulating insulin levels, high dosages of WPF and FPF treatments elicited dissimilar effects on circulating glucose levels, energy expenditure and caecal microbial populations. These data reveal that the cotyledon and hull components of yellow peas facilitate distinct physiological responses.

Given that both WPF and FPF contain the fibre component of yellow peas, pea-derived fibre is likely the bioactive component that reduced circulating insulin levels. Interestingly, the decrease in insulin levels occurred independent of differences in daily food of intake and body weight; and suggests that the hypoinsulinemic effects of pea-derived fibre is secondary to modulation of glucose metabolism rather than the attenuation of excess body weight gain. Moreover, although animals in the FPF group consumed less energy per day compared to controls, energy intakes of FPF and WPF groups were similar. The present results mirror previous observations in our laboratory, whereby WPF and FPF imposed ≈23% reductions in circulating insulin levels in overweight, hypercholesterolemic humans under controlled dietary conditions that prevented weight loss or gain (Marinangeli & Jones, 2010). The mechanisms by which pea-derived fibre reduces circulating insulin levels cannot be determined from the present data. However, heightened insulin sensitivity would suppress the need for excess insulin secretion as a means of maintaining circulating glucose levels. The majority of fibre found in FPF and WPF is insoluble (**Table 14**); epidemiological data and human clinical trials have demonstrated increased insulin sensitivity with insoluble fibre consumption (de Munter et al., 2007; Robertson et al., 2005; Schulze et al., 2007; Weickert et al., 2006). The insulin sensitizing effects of insoluble fibres have been attributed to short-chain fatty acid (SCFA) production secondary to gastrointestinal fermentation of fibres, as well as modulation of adiponectin, ghrelin, glucose-dependent insulinotropic polypeptide and G-coupled proteins (Robertson, 2007; Robertson et al., 2005; Weickert

et al., 2005; Weickert et al., 2006). Overall, pea-derived fibres modulate circulating insulin levels independent of weight loss.

Compared to controls, hamsters receiving FPF demonstrated reductions in circulating glucose levels. As shown in **Table 14,** fibre is the foremost constituent of FPF and as a result, total available carbohydrate intake of hamsters receiving FPF was 1.54 g/d, compared to 2.52 and 1.85 g/d for Controls and WPF, respectively. Nonetheless, given that hamsters were fasted for 10 hrs prior to blood collection, perturbations in glucose metabolism likely contribute to FPF's glucose-lowering effects. Most studies evaluating effects of fibre on blood glucose levels have focused on post-prandial glycemic response with soluble fibre. Soluble fibre's efficacy in reducing post-prandial glycemia has been attributed to its ability to form a viscous gel in the small intestine and interfere with glucose absorption (Jenkins et al., 1978). Conversely, insoluble fibres have been demonstrated to exert negligible effects on glucose absorption, likely due to their lack of viscosity. Sows consuming high levels of soluble fibre showed a significant reduction in glucose absorption and post-prandial glucose concentrations, while insoluble fibre showed no effect (Serena et al., 2009). The present study evaluated the effects of treatments on circulating glucose levels and did not assess post-prandial responses. Nonetheless, dietary fibres have been shown to have effects on glucose homeostasis that would endure beyond the post-prandial period. Although mechanisms are not completely understood, SCFA have been demonstrated to modulate hepatic glucose metabolism. For example, butyrate is a prominent byproduct of pea fibre fermentation (Stark & Madar, 1993) and has been shown to decrease glycolysis and increase glycogen storage (Anderson & Bridges, 1984; Beauvieux et al., 2008). Moreover, Thorburn et al. (1993) noted an association between SCFA production secondary to the microbial fermentation of soluble fibres and resistant starches, with reductions in hepatic glucose output. Given that SCFA production occurs in the latter stages of digestion, when digesta have reached the large intestine, the effects of modulating hepatic glucose output would likely be observed during the non-feeding period. While speculative, it is possible that this mechanism played a role in our observations. However, we acknowledge that lack of

data concerning SCFA concentrations in the blood and caecum is a limitation of the present study. Unfortunately, all caecal contents were used for determination of microbial populations before SCFA analysis could be pursued.

In addition, insoluble fibres have been shown to modulate glucose homeostasis in humans and given that pea hulls are approximately 69% cellulose (Reichert, 1981), the possible effects of insoluble fibre from fractionated pea flour on circulating glucose levels cannot be ignored. Using stable isotopes, it was shown that insoluble fibre increased the rate of disappearance of glucose rather than modulating its rate of appearance (glucose absorption) (Schenk et al., 2003). Similar results were demonstrated by Pouteau et al. (2010) using soluble acetogenic fibre. In the present study, hamsters consuming FPF received ≈825 mg fibre/d as 630 mg/d insoluble fibre and 195 mg/d soluble fibre. Thus, compared to WPF (≈167 mg total fibre/d, 148 mg insoluble, 19 mg soluble) the daily dosage of FPF could have provided optimal levels of insoluble and soluble fibre needed to modulate glucose homeostasis by enhancing hepatic and skeletal glucose uptake, glycogen storage and reduced hepatic glucose secretion.

The observed increase in oxygen consumption in hamsters receiving WPF suggests an increase in energy expenditure. Although WPF contains less fibre compared to FPF, a large proportion of WPF consists of protein and available carbohydrate (**Table 14**). Previous studies have demonstrated that protein provides the highest thermic effect of food given that more energy is required to catabolize dietary proteins into amino acids, synthesize new proteins and produce glucose via gluconeogenesis (Krebs et al., 2003; Robinson et al., 1990). In addition to protein, carbohydrate oxidation is relatively plastic in response to fluxes in dietary carbohydrate intake. In situations of acute overfeeding, the majority of excess dietary carbohydrate is oxidized as well as stored as glycogen, with relatively low levels of carbohydrate being utilized for de novo lipogenesis (Acheson et al., 1982; Acheson et al., 1988; Hellerstein et al., 1996). However, an evaluation of macronutrient intake reveals that hamsters consuming control and WPF

diets had similar intakes of protein at 1.94 and 1.92 g/d, respectively. Moreover, available carbohydrate intake was higher for controls (2.52 g/d) versus the WPF group (1.85 g/d). The current data do not delineate how WPF increased oxygen consumption. However, using previously published nutritional composition data (Pownall et al., 2010; Sales et al., 1995) we determined that daily arginine intake was ≈24% greater amongst hamsters consuming WPF compared to controls at 60.3 and 48.5 mg/d, respectively. Arginine has been identified as an anti-obesity amino acid secondary to its ability to increase carbohydrate oxidation (McKnight et al., 2010). Unfortunately, after our analysis of data from energy expenditure measurements, an error in the collected CO_2 data noted and prevented the calculation of respiratory quotient values and total energy expenditure. Nonetheless, the accuracy and precision of O_2 data was confirmed and suggests that WPF can modulate energy expenditure and justifies further research into its thermogenic properties.

Dual energy X-ray absorptiometry analysis revealed that FPF and WPF tended to have lower fat mass compared to controls. However, no differences in fat mass were observed once body weight gain and energy intake were included as covariates in the statistical analysis. Although not significantly different, percent body weight gain amongst FPF and WPF were 50% less than the control group. High intakes of insoluble fibre have been shown to increase the expression of transcription factors that modulate fat oxidation. Using mice, Isken et al. (2010) demonstrated that, compared to soluble fibre, insoluble fibre decreased energy digestion and body weight and tended to reduce body fat. In addition, mice fed insoluble fibre saw an increase in the expression of transcription factors that facilitate triglyceride oxidation including peroxisome proliferator-activated receptor (PPAR)-α, forkhead transcription factor, and PPAR-γ coactivator β (Isken et al., 2010). The expression of the enzyme diacylglycerol acetyltransferase-2 was also increased and liver homogenates demonstrated less triglyceride deposition (Isken et al., 2010). It is unknown why total body lipid deposition was not modulated in the present study given the high doses of insoluble fibre consumed by the FPF group. Moreover, mice in the Isken et al. study were mature, while the hamsters in the present study were in

their growth phase and factors such as circulating hormone levels and/or disparities in diet could have interfered with the effect of insoluble fibre on fat deposition.

Finally, the second objective of the present study was to investigate whether WPF and/or FPF modulate caecal microbial populations. Data indicate that the most substantial changes in the caecal microbiome occurred within the phylum Firmicutes. Chi-square tests of independence coupled with Fisher's Exact Test revealed that WPF produced a higher abundance of unclassified Clostridia compared to controls and FPF, respectively. Recent studies show that the abundance of Firmicutes and Clostridia are significantly reduced in type II diabetics compared to non-diabetes (Larsen et al., 2010). Recent metagenomic data identified Firmicutes as a dominant microbial group within healthy gastrointestinal tracts at 49% of colonic microflora in healthy patients compared to 22% in Crohn's disease patients (Manichanh et al., 2006). In addition, Firmicutes, including Clostridia, are prominent fibre degraders. Analysis of environmental gene tags suggest that Firmicutes house a greater number of glycolytic enzymes compared to Bacteroidetes (Turnbaugh et al., 2006). Moreover, analysis of taxonomic classes and orders revealed that levels of Clostridia in human feces is associated with fibre fermentation in men (Chinda et al., 2004). Given that fibre degradation via fermentation produces bioactive compounds such as SCFA, enhancement of Clostridia could provide benefits to the host. For example, *Roseburia intestinalis*, which is classified under Clostridia, is a butyrate producing microbe (Duncan et al., 2002). Given that butyric acid serves as an energy source for colonic cells (Roediger, 1980) and, as discussed above, improves glucose homeostasis by enhancing glycogen storage (Beauvieux et al., 2008), butyrate production is identified as a beneficial characteristic of "good" bacteria.

This study also demonstrated an increase in the order Bacilli and class Lactobacillales. The genus Lactobacillus is characterized under Lactobacillales and is one of the foremost genera of bacteria added to probiotic formulations. Supplementing with Lactobacillus has demonstrated efficacy in enhancing immune function (Boge et al., 2009), modulating

abdominal obesity (Kadooka et al., 2010), managing arthritis (So et al., 2008) and preventing diverticular disease (Lamiki et al., 2010), as well as nosocomial diarrhea secondary to *Clostridium difficile* infection (Gao et al., 2010). Future studies employing a more intricate analysis of colonic microbial populations will shed light on specific beneficial metabolic effects of prebiotic WPF and FPF.

Current data are unable to determine if changes noted at the taxonomic levels of class and order, propagate gastrointestinal health. The present data show that WPF induced a greater effect on the microbiome compared to FPF. The increased effect of WPF on caecal microflora populations could be secondary to the proteins found in WPF given that gastrointestinal microflora have the ability to breakdown amino acids which would facilitate bacterial proliferation and possibly the formation of byproducts that foster the growth of other microflora (Macfarlane et al., 1992). Compared to untreated cultures, pea protein extract and hydrolysates have been shown to facilitate the growth of *Lactobacillus acidophilus* while hampering the growth of *Escherichia coli* (Swiatecka et al., 2010). The observed shift in microbiota after consumption of WPF and FPF amongst hamsters provides a strong rationale that a thorough investigation into the prebiotic effects of whole and fractionated pulse crops is warranted.

Adding WPF and FPF to the diets of hamsters at 10% represented a high pulse-crop diet. For example, for a 70 kg human consuming ½ cups pulse crops/d (130-150 g cooked), pulse-crop consumption can be calculated as 0.2% body weight. Hamsters in the present study consumed ≈ 0.97 g/d WPF and FPF. Normalized to body weight, pulse consumption in hamsters was 0.8% body weight and was 300% greater than the corresponding recommended dose in a 70 kg human. While it is unlikely that humans would be able to consume equivalent amounts WPF and/or FPF, results from the present study support the need for dose response studies that evaluate the physiological benefits of pulse crops.

6.6 CONCLUSION

In conclusion, results from the present study have demonstrated that high doses of whole and fractionated yellow pea flour reduce insulin levels independent of changes in body weight and food intake. Moreover, FPF imposed significant reductions in circulating glucose levels, while WPF increased energy expenditure. In addition, the present study presents preliminary data showing that WPF elicits shifts in caecal microbial populations and serves as rationale for further investigation into the prebiotic effects of whole and fractionated pulse crops. However, high doses of WPF and FPF did not modulate circulating cholesterol and triglyceride levels in Golden Syrian hamsters. Nonetheless, the observation that whole and fractionated yellow peas differentially modulate fasting glucose, energy expenditure and caecal microbiota support the notion that different fractions of pulse crops, including bioactivity and dose, should be investigated as functional food ingredients. Results from the present study suggest that that distinct constituents of pulse crops can be recommended as dietary interventions to prevent or manage disease.

6.7 ACKNOWLEDGMENTS

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BRIDGE TO CHAPTER VII

The data generated from the animal trial described in Chapter VI reveal that whole and fractionated yellow pea flour modulate the gastrointestinal microbiome of Golden Syrian hamsters. In addition, analyses of caecal digesta show that whole and fractionated yellow pea flours impose distinct effects on the abundance of specific populations of gastro-microbiota. Given that whole yellow peas, including the cotyledon and hull, are ≈50% available carbohydrate, ≈20% protein and ≈20% fibre, while fractionated yellow peas (hulls only) are ≈85% fibre, the ability for whole and fractionated yellow pea flours to impart distinct effects caecal microflora is likely secondary to differences in nutritional composition. However, it remains to be determined whether whole and fractionated yellow pea flours elicit similar results in humans using USDA recommended dosages.

Compared to other species, diets of humans demonstrate considerable variation. Perturbations in diet during active study phases could modulate gastrointestinal microflora populations and compramise the prebiotic effects of dietary interventions. Thus, strict diet and energy controlled paradigms are essential in human clinical trials that investigate the effects of prebiotic ingredients on the colonic microbiome.

Using an energy and diet controlled paradigm alongside USDA recommended dosages of ½ cup/d pulse crops, the objective of Chapter VII was to delineate whether whole and fractionated yellow pea flours modulate the human gastrointestinal microbiome. The following study could provide further support for whole and fractionated pulse crops as healthy functional ingredients.

CHAPTER VII

WHOLE AND FRACTIONATED YELLOW PEA FLOURS MODULATE THE GASTROINTESTINAL MICROBIOME

Christopher P.F. Marinangeli[1], Ehsan Khafipour[2], Fuqin Zhu[2], Dennis Krause[1,2], and Peter J.H.Jones[1]

[1] University of Manitoba, Richardson Centre for Functional Foods and Nutraceuticals, 196 Innovation Drive, Winnipeg, Manitoba, R3T 2N2, Canada

[2] University of Manitoba, Department of Animal Science, Animal Science Building Winnipeg, Manitoba, R3T 2N2, Canada

To whom correspondence should be directed:

Dr. Peter J.H. Jones, PhD

Richardson Centre for Functional Foods and Nutraceuticals

University of Manitoba

196 Innovation Drive

Winnipeg, MB, CANADA

R3T 2N2

Tel: +1 204 474 8883

Fax: +1 204 474 7552

Email: peter_jones@umanitoba.ca

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7.1 ABSTRACT

Whole and fractionated yellow peas possess distinct nutritional compositions. The objective of the present study was to determine if whole and fractionated yellow peas induce prebiotic effects when administered at the USDA's recommended dose of ½ cup/d pulse crops. Using a crossover design, 23 overweight men and women engaged in three 28 d treatment phases where diet composition and energy intake were controlled to prevent nutrition and weight fluctuations. Treatments included ½ cup/d whole yellow pea flour (WPF), fractionated yellow pea flour (FPF) and a control (white wheat flour, WF). During week four of each phase, two fecal samples were collected from each subject. Pyrosequencing analysis revealed that both WPF and FPF induced increases ($p<0.001$) in the phyla of Proteobacteria, Bacteroidetes, and Firmicutes as well as genera within each phylogenic group including Bacteroides, Pseudobutyrivibrio, Roseburia, Faecalibacterium, and Ruminococcus. In addition, multivariate discriminant analysis revealed that FPF and WPF induced greater diversity of phyla and genera compared to WF. Finally, quantitative PCR demonstrated that FPF and WPF tended to increase ($p<0.1$) Lactobacillus and Bifidobacterium, respectively. The present study demonstrates that whole and fractionated yellow peas induce similar as well as distinct prebiotic effects and provides further support for pulse crops serving as functional food ingredients that promote health and wellbeing.

7.2 INTRODUCTION

Recent insights into the gastrointestinal microbiome demonstrate that microflora residing in the human colon can modulate local as well as systemic inflammation, immunity, intestinal function and metabolism (Beauvieux et al., 2008; Marco et al., 2006; Ng et al., 2009; Sherman et al., 2009; Shida & Nanno, 2008; Tappenden et al., 2003). Given that scientific evidence supports the presence of a symbiotic relationship between gastrointestinal microflora and the host, dietary interventions that induce positive shifts in the colonic microbiome continue to be explored. Most dietary interventions aimed at modulating intestinal microbial populations utilize probiotics. That is, the ingestion of one or more strains of viable bacteria that have demonstrated an ability to induce a physiological benefit to the host (Fuller, 1991). Alternatively, fewer studies have investigated the effects of novel prebiotics. Prebiotics are undigested components of food that selectively increase the growth of one or more types of colonic microflora which in turn elicits a benefit to the host (Gibson & Roberfroid, 1995). Fibres are the most predominant food component that avoids digestion in the small intestine. Consequently, fibres such as oligofructoses, resistant starches and modified celluloses are the most common prebiotic ingredients. Recently, mice consuming a high fat diet alongside prebiotic oligofructose demonstrated a selective increase in Bifidobacterium. In addition, enhanced levels of Bifidobacterium have been credited with preventing the metabolic endotoxemia demonstrated in mice consuming high fat diets alone and with cellulose (Cani et al., 2007).

Pulse crops including lentils, beans, chickpeas, and peas are excellent sources of fibre, and thus, are ideal prebiotic ingredients given that they require little-to-no post-harvest processing. However, the effects of pulses as prebiotic ingredients remain undetermined. In addition, pulses are high in protein and amino acids which can also modulate the colonic microbiome. Therefore, different fractions of pulse crops could induce different prebiotic effects. Yellow peas are ideal for assessing the prebiotic nature of different

components of pulse crops given the ease to which they can be fractionated. Nutrient analyses reveal that the primary source of fibre in the yellow pea is within the outer hull at ≈ 84%. Alternatively, the nutrient composition changes when the whole pea, including the hull and cotyledon, is considered at ≈50% available carbohydrate, ≈20% protein and ≈20% fibre. Recognition that components of pulse crops impose distinct prebiotic effects will justify future studies that explore how pulse-crop fractions can be utilized to improve the health of the host by inducing shifts in specific gastrointestinal microbial populations.

Given that the prebiotic benefits of pulse crops remain largely unexplored, beneficial shifts in the intestinal microbiome were not utilized as criteria to setting the USDA's ½ cup/d dietary guidelines. Hence, the question as to whether USDA recommendations are sufficient for pulse crops to facilitate prebiotic effects requires investigation.

Using quantitative PCR, Terminal Restriction Length Polymorphism analysis (TRFL-P) and pyrosequencing, the objective of the present study was to determine whether the USDA's recommended dose of ½ cup/d whole or fractionated yellow peas modulates the gastrointestinal microbiome in overweight humans under controlled dietary conditions.

7.3 MATERIALS AND METHODS

7.3.1 Study Subjects

Using newspaper and radio advertisements, 29 overweight men and women were recruited from the Winnipeg area. The average ages of men and women were 51.8 ± 12.3 yrs and 52.3 ± 10.0 yrs, respectively. The average BMI of men and women was 31.7 ± 5.3 kg/m^2 and 29.4 ± 3.5 kg/m^2, respectively. Given that the present study was an extension of a larger clinical trial investigating the lipid-lowering properties of whole and fractionated yellow peas, inclusion and exclusion criteria include parameters that control

for external modification of circulating lipid levels. Exclusion criteria included smoking, the use of prescription and natural lipid lowering therapies, history of myocardial infarction, coronary artery bypass, angina, congestive heart failure, inflammatory bowel disease, pancreatitis, renal disease, diabetes and a history of chronic alcohol usage (> 2 drinks/d). Individuals with cancer, eating disorders and those who reported expending > 16 747 kJ/week through exercise were also excluded from participation. Participants taking medications for other ailments were included providing their daily dose of medication remained stable for the duration of the study. This study was approved by the University of Manitoba's Biomedical Research Ethics Board (B2006: 129). Written informed consent was obtained from all subjects. This study was registered at www.clincialtrials.gov, ID NCT00839774 (February 6, 2009).

7.3.2 Treatments

Treatments consisted of whole yellow pea flour (WPF), fractionated yellow pea flour (hulls only) (FPF) and white wheat flour (control) (WF). WPF and FPF flours were obtained from Best Cooking Pulses Inc. (Portage la Prairie, MB, Canada), while the WF was obtained from a local supermarket. Prior to milling, yellow peas were dried on the vine and subsequently harvested. Whole yellow peas were milled without further processing. In order to produce FPF, hulls were separated from their cotyledons and subsequently milled into flour. The nutritional composition of WPF and FPF were assessed by Medallion Laboratories (Minneapolis, MN, USA) and is summarized in **Table 18.** The composition of WF was determined by available food analysis tables (Brault Dubuc & Caron-Lahaie, 1994). Treatment and control flours were administered to subjects in muffins that were developed at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) clinical kitchen. Current USDA recommendations for pulse consumption are ½ cup/d, which is ≈ 50 g dry whole yellow peas (USDA.gov, 2009). Each WPF treatment muffin was formulated to contain ≈ 25 g, 50% of the USDA

Table 18. Composition of treatment flours and treatment muffins

Treatment Flour	**WPF**[1§]	**FPF**[1§]	**WF**[2]
Energy (kJ)/100 g	1 281	343	1 482
Total carbohydrate (%)	66.3	89.0	79.4
Available carbohydrate (%)	50.7	12.7	76.3
Fibre (%)	17.6	84.2	3.1
Soluble (%)	2.0	7.9	1.8
Insoluble (%)	15.6	76.3	1.3
Total fat (%)	1.84	0.63	1.0
Polyunsaturated fat (%)	0.98	0.31	0.41
Monounsaturated fat (%)	0.44	0.13	0.09
Saturated (%)	0.34	0.16	0.15
Protein (%)	21.6	6.39	10.3
Treatment Muffins	**WPF**[1]	**FPF**[1]	**WF**[2]
Whole pea flour per muffin (g)	26.4	-	-
Fractionated pea flour per muffin (g)	-	6.0	-
White wheat flour per muffin (g)	-	20.4	26.4
Weight per muffin (g)	91	91	91
Energy (kJ)	821.5	816.9	888.5
Carbohydrate (g)	30.4	33.8	33.0
Total fibre (g)	5.8	6.9	2.0
Soluble fibre (g)	1.7	2.0	1.6
Insoluble fibre (g)	4.1	4.8	0.3
Total fat (g)	7.4	7.1	7.1
Polyunsaturated fat (g)	3.8	3.7	3.7
Monounsaturated fat (g)	3.2	3.1	3.1
Saturated fat (g)	0.9	0.8	0.8
Protein (g)	7.1	3.9	4.1

Abbreviations: WPF, whole pea four; FPF, fractionated pea flour; WF, white wheat flour.

[1] Treatment flours analyzed at Medallion Labs (Minneapolis, Minnesota, USA).

[§] Analysis of total, soluble and insoluble fibres was completed by Medallion Labs using AOAC method 991.43.

[2] Flour composition determined using available nutritional tables (Brault Dubuc & Caron-Lahaie, 1994)

recommended daily serving of pulses. Given that two WPF treatments were equivalent to ½ cup/d dry yellow peas (50 g), subjects were required to eat two treatment muffins/d. The FPF treatment muffins were formulated based on total pea-derived fibre content of the WPF muffin since the pea hull flour used in the present study was approximately 84.2 % fibre (**Table 18**). Hence, the amount of fractionated flour added to the FPF muffin recipe was adjusted to equal the amount of fibre in the WPF treatment (**Table 18)**. The balance of the muffin weight was white wheat flour. Adjusting FPF treatments to contain the same amount of pea fibre as the WPF treatment enabled distinguishing which prebiotic effects can be attributed to cotyledon and/or hull component of yellow peas. The WF muffin was solely comprised of white wheat flour.

7.3.3 Study Design

The present study used a randomized single blind, controlled crossover design. The study consisted of three, four-week study phases, each followed by a four-week washout. To determine the treatment order within each subject, participants were randomly assigned to one of the six possible treatment sequences.

7.3.3.1 Diet and Treatment Protocol

For the duration of each study phase, subjects only consumed food provided by the RCFFN clinical research kitchen. The nutrient composition of the study diet was analyzed using Food Processor version 7.71 (ESHA Research, OR, USA) as is summarized in **Table 19**. Diets did not contain any pulse products besides the treatment muffins. To prevent bodyweight change for the duration of each study phase, daily energy requirements for each subject were determined by calculating individual resting metabolic rates using the Mifflin Equation (Mifflin et al., 1990), then applying an activity factor of 1.7. The amount of food each participant received was based on individual

calculated energy requirements. The average caloric content of two treatment muffins (approximately 1,675 kJ) was subtracted from the total calculated energy requirements to compensate for the energy intake from WPF, FPF and WF. Body weights were assessed daily. If a subject's weight increased or decreased in the first week of each study phase, energy intake was adjusted accordingly. Each morning, study participants were required to visit the RCFFN Clinical Research Unit to consume breakfast and one banana flavored treatment muffin under supervision. Participants would then leave the unit with a cooler containing the remainder of their meals and snacks alongside an apple-flavored treatment muffin to consume with dinner. Returned empty, unwashed food containers were used to monitor compliance.

Table 19. Energy requirements and background diet composition for study participants

Diet Composition	
Carbohydrate (%)	55%
Protein (%)	15%
Fat (%)	30%
Diet Summary[§]	
Energy (MJ/d)	12.56
Carbohydrate (g)	403.7
Protein (g)	113.2
Fat (g)	97.3
Polyunsaturated fat (g)[¥]	19.6 (6%)
Monounsaturated fat (g)[¥]	20.8 (6%)
Saturated fat (g)[¥]	25.1 (8%)
Dietary fibre (g)	18.8
Soluble fibre (g)	4.8
Cholesterol (mg)	238.8

[§] Nutritional summary is based on a 12.56 MJ/d diet

[¥] Brackets denote percent of total energy

7.3.3.2 Fecal Collection Protocol

On the Wednesday and Friday of week four of each study phase, fecal samples were collected from subjects. Sampling was completed by volunteers in the privacy of their own home. On the morning of the day that samples were to be collected, commodes were distributed to subjects alongside fecal collection sampling and storage apparatus as well as written instructions. Subjects were only required to provide a sample from one bowel movement. If volunteers were unable to provide a sample on the required day, they were instructed to collect a sample from their next bowel movement on the following day. After collections, subjects were instructed to store samples in their freezer and return them to the clinical research unit the following morning. Samples were subsequently stored at -80°C until analysis.

7.3.4 Prebiotic Analysis of Fecal Samples Analysis

7.3.4.1 Fecal DNA Extraction

DNA from fecal samples was extracted using ZR Fecal DNA Kit (D6010, Zymo Research Corp., Orange, CA, USA). The bead beating step utilized to mechanically lyse cells was extended to 3 min. Quality of DNA was evaluated using gel electrophoresis.

7.3.4.2 Terminal Restriction Length Polymorphism Analysis

PCR amplification of DNA samples was conducted using previously described methodologies (Khafipour et al., 2009; Sepehri et al., 2007). Briefly, V1 and V2 regions of ribosomal DNA (rDNA) genes were amplified using universal bacterial primers specific for prokaryotic sequences, 27f (5'-AAGAGTTTGATCATGGCTCAG-3') and 342r (5'-TGCTGCCTCCCGTAG-3'). To allow for detection during electrophoresis, the

forward primer was fluorescently labeled (WellRED D4dye, Sigma-Proligo, St. Louis, MO, USA) (Khafipour et al., 2009). The PCR amplification program was as follows, one cycle at 94°C for five min; then 36 cycles at 94°C for one min; 56°C for one min; 72°C for two min; and a final extension at 72°C for five min.

Terminal restriction fragments were generated by digesting PCR products with *Hha*I (15 µL of PCR product, 10 units of *Hha*I, 1X *Hha*I buffer, and 20 µg of bovine serum, New England Biolabs, Ipswich, MA) at 37°C for three hours. Fragment lengths were determined using a CEQ 8800 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA). Two µl of fluorescently labeled PCR product alongside 29.5 µL of sample loading solution, and one µL of 600 basepair (bp) DNA standard (Beckman Coulter, Inc., Fullerton, CA) were mixed for analysis. An electropherogram with variable peak sizes was generated for each within treatment pooled DNA sample. Peaks of different sizes corresponded to disparate operational taxonomic units (OTU). Fragments were analyzed using CEQ software (Version 9.0; Beckman Coulter Inc., Fullerton, CA) using a two bp binning parameter. Only fragments that constituted > 1% total abundance were included in the analysis. Data from the OTU profiles were grouped based on treatment and sampling time and used numerical analysis. Data are presented as percent abundance of microbial populations within Phyla and further segregated into the taxa, class and order.

7.3.4.3 Quantitative PCR Analysis

The primers utilized for quantitative PCR were assembled from the literature and are listed in **Table 20.** Real-time PCR was carried out using an AB 7300 system (Applied Biosystems, Foster City, CA, USA) and sequence detection software (Version 1.3; Applied Biosystems, Foster City, CA, USA). Each reaction was run in triplicate in a volume of 25 µl in optical reaction plates (Applied Biosystems, Foster City, CA, USA) sealed with optical adhesive film (Applied Biosystems, Foster City, CA). Amplification reactions were carried out with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) mixed

with the selected primer set at a concentration of 0.5 μ*M* for each primer, and 2 μL (~12 ng) of genomic DNA. Amplification was consisted of one cycle of 95ºC (10 min), and 40 cycles of denaturation at 95ºC (15 sec), and annealing at 60ºC (1 min). Final melting analysis was obtained by slow heating from 65ºC to 95 ºC. To evaluate the efficiency (E) of the amplification of each primer set, DNA templates were pooled (50 ng/reaction) and serially diluted 8 fold. Amplification efficiency (E) was calculated from the slope of the standard curve generated from the plotting of the threshold cycle (CT) versus logarithmic values of different DNA concentrations using the following equation (Pfaffl, 2001):

$$E = 10^{\frac{-1}{slope}}$$

Relative quantitation was accomplished using the Pfaffl mathematical model (Pfaffl, 2001):

$$R = \frac{E_{\text{target}}{}^{CT_{\text{target}}}}{E_{\text{ref}}{}^{CT_{\text{ref}}}}$$

Where R is the relative expression ratio of a target gene compared to a reference gene, E is the amplification efficiency, *target* is the 16S rDNA gene of species of interest, *ref* is the 16S rDNA gene of *Eubacteria*.

7.3.4.4. Pyrosequencing Analysis

The same extracted DNA utilized for TRFL-P analysis was utilized for pyrosequencing. Pyrosequencing analysis was conducted by Research and Testing Laboratories (Lubbock, TX, USA). Data presented from pyrosequencing analysis represent the number unique sequences detected that belong to specific phyla and genus. Given that pyrosequencing analysis generated >310 000 unique sequences spread across 12 phyla and 210 genera, microbial populations corresponding to > 0.1% total abundance underwent statistical analysis to determine if WF, WFP and FPF yielded significant variation in the intestinal microbiome.

Table 20. List of primers used for quantitative PCR of microbial genera from feces-derived DNA

Target group	Primer Set[§]	Primer Sequences	Amplicon Size (bp)
Eubacteria	341-357F	CCTACGGGAGGCAGCAG	189
	518-534R	ATTACCGCGGCTGCTGG	
Lactobacillus spp. (Lac)	Ulac16S1F	AGCAGTAGGGAATCTTCCA	345
	Ulac16S1R	ATTCCACCGCTACACATG	
Bacteroides spp. (Bact)	Bac303F	GAAGGTCCCCCACATTG	103
	Bfr-Fmrev	CGCKACTTGGCTGGTTCAG	
Roseburia spp. and E.rectale (Rrec1)	RrecF	GCGGTRCGGCAAGTCTGA	81
	Rrec630mR	CCTCCGACGCTCTAGTMCGAC	
Clostridial cluster IV (Clep)	Clep866mF	TTAACACAATAAGTWATCCACCTGG	314
	Clept1240mR	ACCTTCCTCCGTTTTGTCAAC	
Cluster IV Ruminococcus spp. (Rum)	Rflbr730F	GGCGGCYTRCTGGGCTTT	157
	Clep866mR	CCAGGTGGATWACTTATTGTGTTAA	
Bifidobacterium adolescentis (Bad)	Bif164F	GGGTGGTAATGCCGGATG	298
	BiADO-2	CGAAGGGCTTGCTCCCAGT	

[§]Primers utilized for quantitative PCR analysis were from Ramirez-Farias et al. (2009)

7.3.5 Statistical Analysis

SAS version 9.2 (Cary, NC, USA) was used for all statistical analysis. Pyrosequencing data were analyzed using proc glimmix alongside a poisson distribution. Treatment and phase were used as fixed factors while subject ID was used as a random variable. Tukey's post-hoc test was used to determine which groups differed from each other. For all analysis, $p<0.05$ was used to indicate statistical significance. Finally, JMP 7.0 (SAS, Cary, NC, USA) was used to conduct quadratic multivariate discriminant analysis to determine relationships between treatments and specific phyla and genus populations.

7.4 RESULTS

Two subjects dropped out for medical reasons while four dropped out for personal reasons. Of the 23 volunteers who completed the 12 wk trial, 16 were women and seven were men. No subjects consumed antibiotics during active study phases.

7.4.1 Terminal Restriction Length Polymorphism and Quantitative PCR Analysis

Terminal restriction length polymorphism data demonstrated no significant difference in abundance amongst microbial phyla and class between WF, WPF and FPF (**Table 21)**. However, quantitative PCR analysis demonstrated that compared to WF, FPF and WPF tended to increase ($p<0.1$) the relative abundance of Lactobacillus and Bifidobacterium, 157 and 338%, respectively (**Table 22**).

7.4.2 Pyrosequencing

Post-data compression, 5 phyla and 29 genera underwent statistical analysis and are shown in **Figure 12** and **Tables 23 & 24**. Although the abundance of bacteria within the genera Bradyrhizobium, Turicibacter and Sphingomonas represented >0.1% total abundance, the aforementioned genera were not analyzed given that data were unable to converge using various distributions during statistical analysis.

Table 21. TRFL-P analysis of fecal microbiota

Microbial level, %	Treatments		
	WPF	**FPF**	**WF**
Phylum Actinobacteria	**1.165 ± 0.172**	**1.04 ± 0.211**	**0.866 ± 0.173**
Class Actinobacteria	1.165 ± 0.172	1.04 ± 0.211	0.866 ± 0.173
Subclass Coriobacteridae	0.592 ± 0.093	0.587 ± 0.135	0.471 ± 0.090
Subclass Actinobacteridae	0.572 ± 0.112	0.453 ± 0.096	0.395 ± 0.105
Phylum Bacteroidetes	**0.872 ± 0.271**	**1.025 ± 0.276**	**0.773 ± 0.082**
Class Flavobacteria	0.162 ± 0.029	0.189 ± 0.016	0.137 ± 0.026
Class Sphingobacteria	0.067 ± 0.021	0.072 ± 0.015	0.069 ± 0.013
Class Bacteroidetes	0.406 ± 0.221	0.488 ± 0.313	0.311 ± 0.141
Phylum unclassified Bacteroidetes	**0.237 ± 0.041**	**0.276 ± 0.040**	**0.256 ± 0.046**
Phylum Tenericutes	**0.137 ± 0.033**	**0.15 ± 0.028**	**0.164 ± 0.020**
Class Mollicutes	0.137 ± 0.033	0.15 ± 0.028	0.164 ± 0.020
Phylum Fusobacteria	**0.025 ± 0.015**	**0.039 ± 0.017**	**0.032 ± 0.017**
Class Fusobacteria	0.025 ± 0.015	0.039 ± 0.017	0.032 ± 0.017
Phylum Deinococcus-Thermus	**0.019 ± 0.002**	**0.02 ± 0.001**	**0.019 ± 0.017**
Class Deinococci	0.019 ± 0.002	0.02 ± 0.001	0.019 ± 0.017
Phylum Proteobacteria	**6.992 ± 1.191**	**6.757 ± 0.826**	**6.982 ± 0.695**
Class Epsilonproteobacteria	0.546 ± 0.077	0.576 ± 0.071	0.539 ± 0.065
Class Deltaproteobacteria	0.609 ± 0.197	0.878 ± 0.136	0.868 ± 0.161
Class unclassified Deltaproteobacteria	0.015 ± 0.005	0.018 ± 0.007	0.021 ± 0.006
Class Alphaproteobacteria	0.829 ± 0.133	0.987 ± 0.048	0.925 ± 0.137
Class unclassified Alphaproteobacteria	0.081 ± 0.017	0.094 ± 0.015	0.068 ± 0.014
Class Gammaproteobacteria	2.004 ± 0.488	1.493 ± 0.518	1.797 ± 0.420
Class unclassified Gammaproteobacteria	0.089 ± 0.057	0.141 ± 0.063	0.143 ± 0.069
Class Betaproteobacteria	2.987 ± 0.421	2.801 ± 0.318	2.822 ± 0.176
Class unclassified Betaproteobacteria	0.011 ± 0.006	0.016 ± 0.006	0.014 ± 0.005
Phylum unclassified Proteobacteria	**0.016 ± 0.007**	**0.022 ± 0.006**	**0.031 ± 0.006**
Phylum Deferribacteres	**0.003 ± 0.003**	**0.00 ± 0.00**	**0.004 ± 0.004**
Class Deferribacteres	0.003 ± 0.003	0.00 ± 0.00	0.004 ± 0.004

(Continued on the following page)

(Table 21 continued)

Phylum TM7	**0.012 ± 0.004**	**0.01 ± 0.005**	**0.007 ± 0.004**
Phylum Verrucomicrobia	**7.599 ± 0.813**	**7.841 ± 0.424**	**7.507 ± 0.906**
Class Verrucomicrobiae	7.599 ± 0.813	7.841 ± 0.424	7.507 ± 0.906
Phylum Firmicutes	**82.729 ± 1.793**	**82.616 ± 1.378**	**83.22 ± 1.487**
Class Bacilli	3.638 ± 0.801	2.835 ± 0.624	2.525 ± 0.330
Class Erysipelotrichi	1.363 ± 0.283	1.042 ± 0.351	0.747 ± 0.180
Class Clostridia	77.64 ± 2.788	78.695 ± 1.522	79.9 ± 1.534
Class unclassified_"Clostridia"	2.061 ± 0.477	1.657 ± 0.419	1.821 ± 0.342
Phylum unclassified Firmicutes	**0.087 ± 0.028**	**0.044 ± 0.028**	**0.047 ± 0.021**
Phylum Lentisphaerae	**0.033 ± 0.006**	**0.03 ± 0.007**	**0.03 ± 0.005**
Class Lentisphaerae	0.033 ± 0.006	0.03 ± 0.007	0.03 ± 0.005
Class unclassified Lentisphaerae	0.013 ± 0.005	0.017 ± 0.003	0.012 ± 0.004
Phylum unclassified bacteria	**0.416 ± 0.070**	**0.472 ± 0.121**	**0.395 ± 0.037**

Abbreviations: WPF, whole pea flour; FPF, fractionated pea flour; WF, white wheat flour

Table 22. Relative quantitative PCR results of key microbial groups

Genera	**Effect of Treatments on Relative Quantitation of Genera**		
	WPF	**FPF**	**WF**
Lactobacillus spp.	0.960 ± 0.768^{a}	2.528 ± 2.141^{b}	0.984 ± 0.605^{a}
Roseburia spp and Eubacterium rectale (subgroup XIVa)	0.849 ± 0.167	0.815 ± 0.173	0.627 ± 0.091
Bacteroides-Prevotella group	0.846 ± 0.116	1.028 ± 0.150	0.935 ± 0.244
Clostridium cluster V (Clostridium leptum)	1.184 ± 0.073	1.101 ± 0.129	1.199 ± 0.274
Ruminococcus bromii and R. flavifaciens (subgroup XIVa)	0.876 ± 0.160	0.749 ± 0.062	1.541 ± 0.647
Bifidobacterium adolescentis	1.836 ± 0.840^{b}	0.571 ± 0.289^{a}	0.419 ± 0.159^{a}

Abbreviations: WFP, whole pea flour; FPF, fractionated pea flour; WF, white wheat flour

Results are expressed as the relative ratio of Log_{10} values (Mean±SEM) genera to total bacterium

Values with different letter subscripts are different from each other ($p < 0.1$)

Figure 12. Taxonomic tree outlining identified by pyrosequencing representing >0.1% abundance of fecal microbiota.

Pyrosequencing analysis of phyla demonstrate that WPF and FPF decreased ($p<0.001$) the number of unique sequences 5 and 4% within Actinobacteria compared to WF, respectively (**Table 23)**. Compared to WF (511.0 ± 97.9 unique sequences), FPF (609.4 ± 116.8 unique sequences) and WPF (693.1 ± 132.8 unique sequences) increased ($p<0.001$) the abundance of Bacteroidetes 19 and 36% respectively. FPF (2899.1 ± 235.4 unique sequences) and WPF (3173.1 ± 257.6 unique sequences) increased ($p<0.001$) the number of unique sequences within Firmicutes compared to WF (2792.3 ± 226.7 unique sequences). Finally, the number of unique sequences within Proteobacteria were increased 63 and 67% ($p<0.001$) in FPF relative to WPF and WF, respectively.

Within each phylum, substantial shifts in microbial populations amongst genus populations were observed between FPF, WPF and FPF. Results are summarized in **Table 24.** The most substantial changes in abundance were noted within Bacteroides, Eubacterium, Roseburia, Faecalibacterium and Ruminococcus. The abundance of Bacteroides for WF (396.8±77.9 unique sequences) was decreased ($p<0.001$) 26 and 16% compared to WPF (541.4±106.2 unique sequences) and FPF (474.2±93.1 unique sequences), respectively. WPF (635.8±89.3 unique sequences) and FPF (578.5±81.3 unique sequences) increased ($p<0.001$) levels of Eubacteria 41 and 28% compared to WF (450.5±63.3 unique sequences). In addition, the abundance of Eubacteria was 10% higher ($p<0.001$) in WPF compared to FPF. Levels of Roseburia were increased ($p<0.001$) 17% in FPF (134.8±21.2 unique sequences) compared to WF (76.3±12.0 unique sequences) while FPF (273.8±112.2 unique sequences) and WPF (247.4±101.3 unique sequences) lowered ($p<0.001$) the abundance of Faecalibacterium 32 and 38% compared to WPF (401.6±164.5 unique sequences), respectively. Lastly, levels of Rumincoccus were lowered ($p<0.001$) 17 and 12% with WPF and WF compared to FPF.

Multivariate discriminant analysis assesses the dissimilarity between treatment groups while indicating the impact of treatments on bacterial phyla and genera. Discriminant analysis of phyla revealed considerable similarity between WF, WPF and FPF (**Figure**

13). However, FPF and WPF demonstrated the greatest effects on Firmicutes and Bacteroidetes. Conversely, WF had a large effect on levels of Actinobacteria. Discriminant analysis of genera within the phylum Bacteroidetes also revealed similarity between WPF, FPF and WF (**Figure 14**). Nonetheless, WPF facilitated greater diversity of genus populations by favoring growth Bacteroides, Prevotella and Alistipes. Finally, discriminant analyses of genera within the phylum Firmicutes demonstrated dissimilarity between treatments (**Figure 15**) and, similar to Bacteroidetes, both FPF and WFP facilitated an increase in diversity amongst Firmicute-derived genera.

Table 23. Pyrosequencing analysis of phyla derived from fecal microbiota representing >0.1% total microbial abundance

Phyla	Effect of Treatment on Number of Unique Sequences			*P value*[§]
	WPF	FPF	WF	
Actinobacteria	9.8 ± 2.5^{a}	$14.8 \pm 3.7^{b**}$	$18 \pm 4.5^{c¥¥, ¶¶}$	<0.001
Bacteroidetes	723.1 ± 128.4^{a}	$682.1 \pm 121.1^{b*}$	$553.8 \pm 98.3^{c¥¥, ¶¶}$	<0.001
Firmicutes	2975.1 ± 274.1^{a}	$3004.9 \pm 276.9^{b**}$	$2755.3 \pm 253.9^{c¥¥, ¶¶}$	<0.001
Proteobacteria	26 ± 6.5^{a}	$24.2 \pm 6.1^{b**}$	$24.8 \pm 6.2^{c¥¥, ¶¶}$	0.314
Verrucomicrobia	0.7 ± 0.4^{a}	$0.9 \pm 0.5^{a**}$	$1.4 \pm 0.8^{b¥¥,¶}$	0.001

Abbreviations: WPF, whole pea flour; FPF, fractionated pea flour; WF, white wheat flour

Mean±SEM

Values with different letter subscripts are significantly different from each other

WPF vs. FPF: $^{*}p<0.05$, $^{**}p<0.001$

WPF vs. WF: $^{¥}p<0.05$, $^{¥¥}p<0.001$

FPF vs. WF: $^{¶}p<0.05$, $^{¶¶}p<0.001$

Table 24. Pyrosequencing analysis of genera from fecal microbiota representing >0.1% total microbial abundance

Phyla and Genera	Effect of Treament on Unique Counts of Sequences			*P value*[§]
	WPF	FPF	WF	
Actinobacteria				
Collinsella	3.0 ± 1.5[a]	3.2 ± 1.6[a]	3.9 ± 2.0[b¥, ¶]	0.013
Bacteroidetes				
Parabacteroides	17.0 ± 7.5[a]	11.5 ± 5.1[b**]	12.9 ± 5.7[c¥¥, ¶]	<0.001
Bacteroides	541 ± 106.2[a]	474.2 ± 93.1[b**]	396.8 ± 77.9[c¥¥, ¶¶]	<0.001
Prevotella	1.5 ± 0.9[a]	2.1 ± 1.3[b**]	0.7 ± 0.4[c¥¥, ¶¶]	<0.001
Alistipes	27.2 ± 8.3[a]	18.6 ± 5.7[b**]	25.1 ± 7.7[a¶¶]	<0.001
Firmicutes				
Lactococcus	1.5 ± 0.3[a]	2.2 ± 0.5[a]	3.1 ± 0.6[b¥¥, ¶¶]	0.001
Streptococcus	5.2 ± 1.2[a]	11.4 ± 2.6[b**]	10.1 ± 2.3[b¥¥]	<0.001
Clostridium	538.7 ± 57.9[a]	549.1 ± 59.0[a]	588.4 ± 63.2[b¥¥, ¶¶]	<0.001
Eubacterium	635.8 ± 89.3[a]	578.5 ± 81.3[b**]	450.5 ± 63.3[c¥¥, ¶¶]	<0.001
Lachnospira	14.6 ± 3.2[a]	16.2 ± 3.6[a]	11.7 ± 2.6[c¥, ¶¶]	0.001
Coprococcus	10.4 ± 2.6[a]	13.0 ± 3.3[b*]	9.5 ± 2.4[a¶¶]	0.001
Dorea	17.0 ± 3.8[a]	22.5 ± 5.0[b**]	17.8 ± 4.0[a¶¶]	<0.001
Pseudobutyrivibrio	5.2 ± 1.3[a]	4.6 ± 1.1[a]	1.9 ± 0.5[b¥¥, ¶¶]	<0.001
Roseburia	108.2 ± 17.0[a]	134.8 ± 21.2[b**]	76.3 ± 12.0[c¥¥, ¶¶]	<0.001
Anaerofilum	7.3 ± 2.1[a]	4.6 ± 1.3b[**]	6.3 ± 1.8[a¶]	<0.001
Ethanoligenens	1.5 ± 0.8[a]	1.2 ± 0.6b[*]	1.4 ± 0.7[ab]	0.026
Faecalibacterium	401.6 ± 164.5[a]	273.8 ± 112.2[b**]	247.4 ± 101.3[c¥¥, ¶¶]	<0.001
Ruminococcus	482.8 ± 80.8[a]	411.6 ± 68.9[b**]	462.4 ± 77.4[c¥¥, ¶¶]	<0.001
Sporobacter	8.0 ± 4.1[a]	6.2 ± 3.2[b**]	11.1 ± 5.7[c¥¥, ¶¶]	<0.001
Anaerotruncus	6.8 ± 2.9[a]	3.9 ± 1.7[b**]	1.1 ± 0.5[c¥¥, ¶¶]	<0.001
Succiniclasticum	0.0 ± 0.0[a]	0.1 ± 0.1[b*]	0.1 ± 0.1[b¥]	0.018
Dialister	0.3 ± 0.2[a]	0.3 ± 0.2[a]	0.7 ± 0.6[c¥¥, ¶¶]	<0.001
Phascolarctobacterium	3.3 ± 2.2[a]	1.8 ± 1.2b[**]	1.5 ± 1.0[c¥¥, ¶¶]	<0.001

(Continued on the following page)

(Table 24 continued)

Proteobacteria				
Acidovorax	0.7 ± 0.3^{ab}	0.5 ± 0.2^{a}	$0.8 \pm 0.4^{b¶}$	0.035
Cupriavidus	0.4 ± 0.3^{a}	$0.2 \pm 0.1^{b*}$	0.4 ± 0.2^{ab}	0.033
Escherichia	0.3 ± 0.2^{a}	$0.7 \pm 0.4^{b**}$	$0.5 \pm 0.3^{c¥, ¶}$	0.001
Klebsiella	0.4 ± 0.3^{a}	$0.0 \pm 0.0^{b**}$	$0.1 \pm 0.1^{b¥}$	<0.001
Pseudomonas	1.4 ± 0.7^{a}	$0.3 \pm 0.1^{b**}$	$0.5 \pm 0.2^{c¥¥, ¶}$	<0.001
Verrucomicrobia				
Akkermansia	1.0 ± 0.5	0.9 ± 0.5	1.1 ± 0.6	0.4099

Abbreviations: WPF, whole pea flour; FPF, fractionated pea flour; WF, white wheat flour

Mean±SEM

Values with different letter subscripts are significantly different from each other

WPF vs. FPF: $^{*}p<0.05$, $^{**}p<0.001$

WPF vs. WF: $^{¥}p<0.05$, $^{¥¥}p<0.001$

FPF vs. WF: $^{¶}p<0.05$, $^{¶¶}p<0.001$

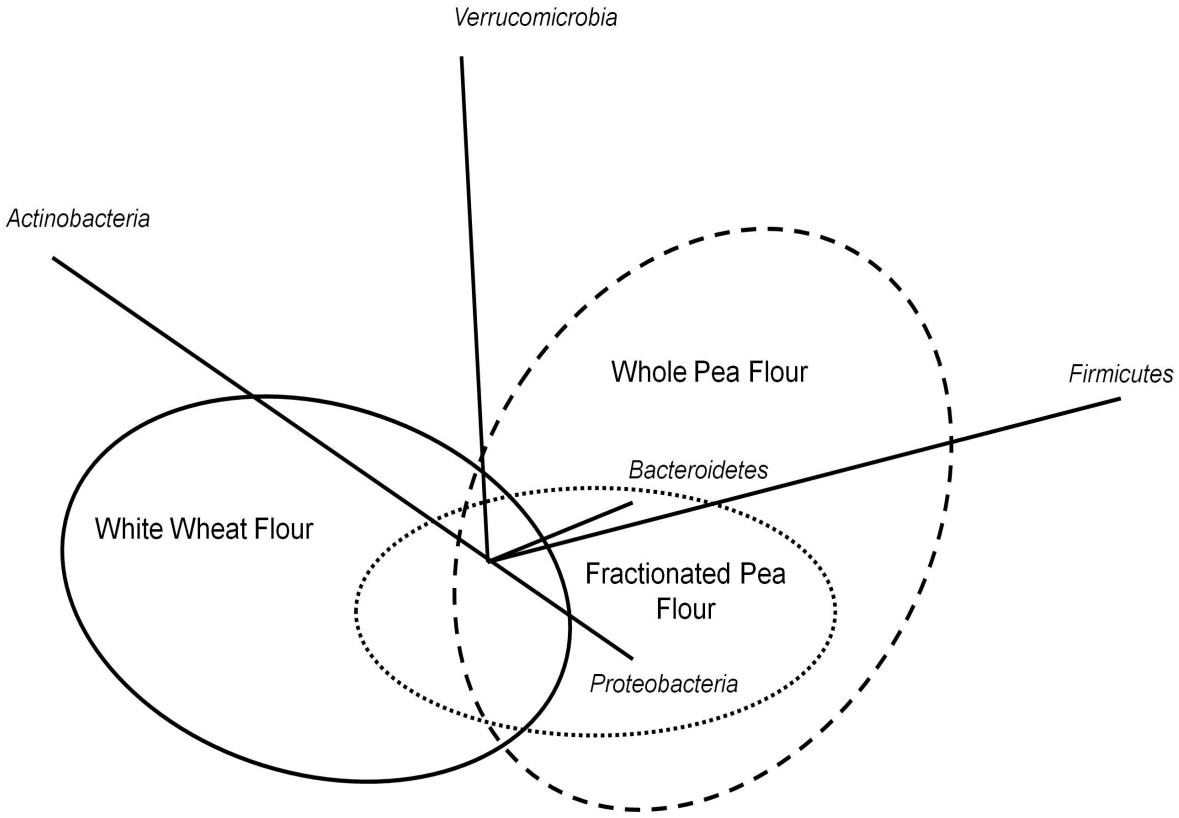

Figure 13. Multivariate discriminant analysis of phyla representing >0.1% total abundance of fecal microbiota as identified by pyrosequencing. Circles represent independent variables and their proximity reflects their dissimilarity. The straight lines are dependent variables, and their lengths and angles between them are functions of the relative effects of independent variables.

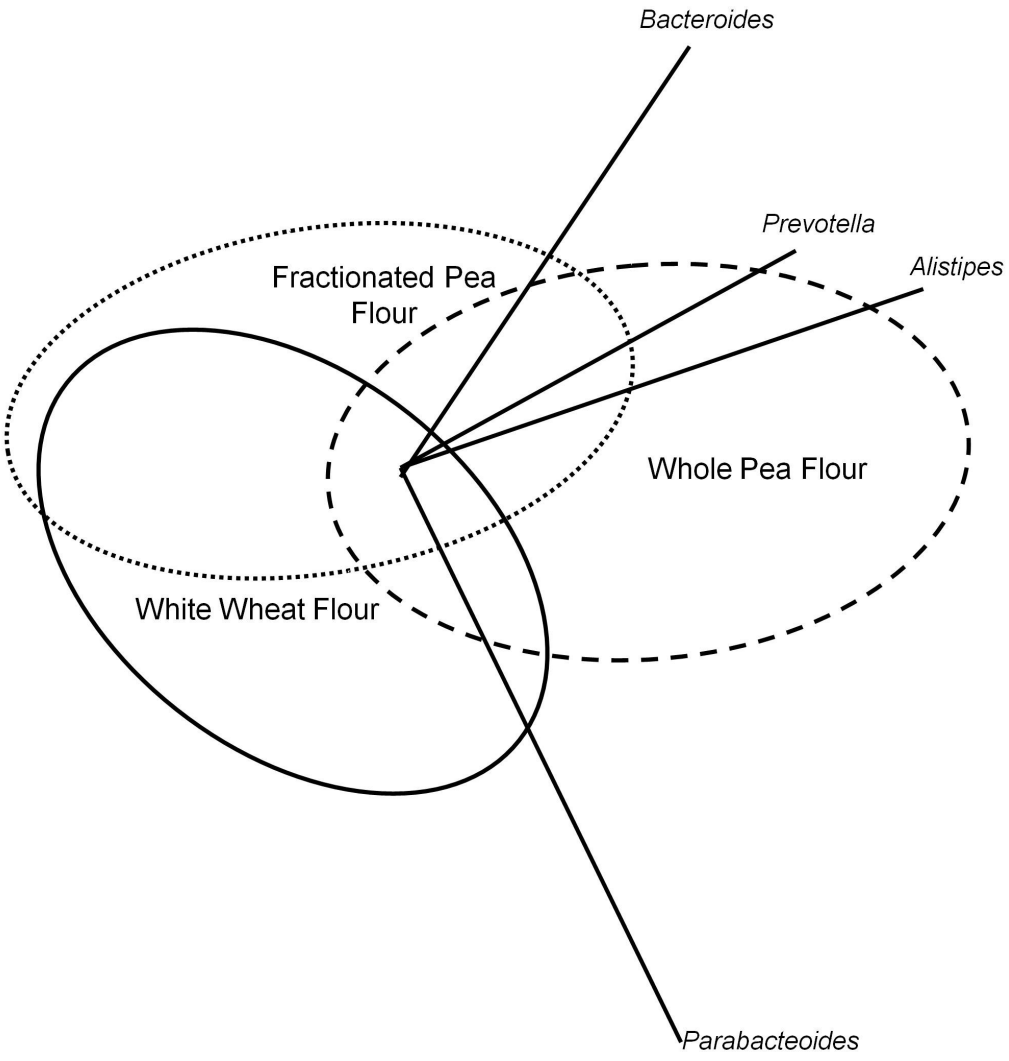

Figure 14. Multivariate discriminant analysis of genera within the phyla Bacteroidetes representing >0.1% total abundance of fecal microbiota as identified by pyrosequencing. Circles represent independent variables and their proximity reflects their dissimilarity. The straight lines are dependent variables, and their lengths and angles between them are functions of the relative effects of independent variables.

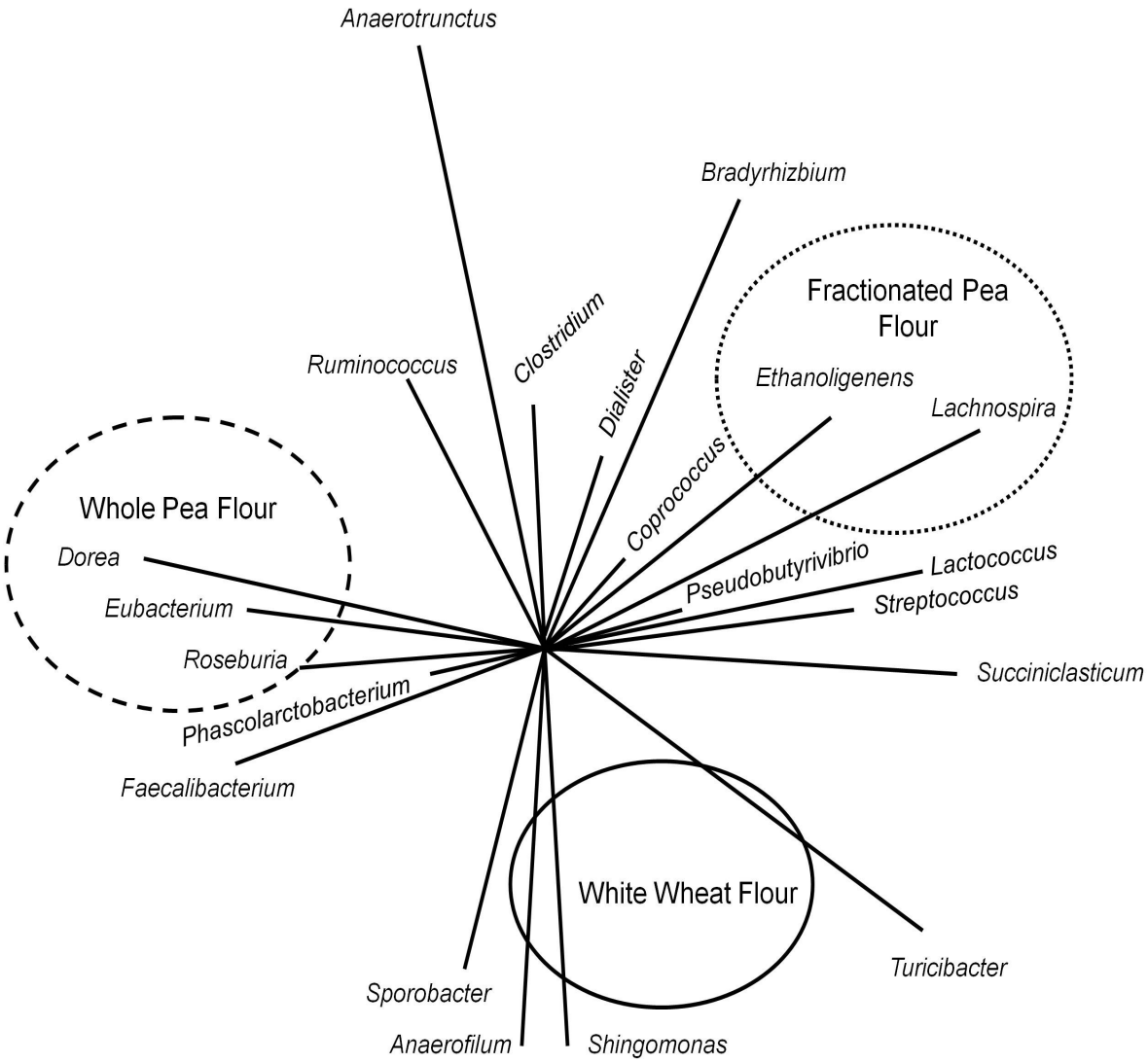

Figure 15. Multivariate discriminant analysis of genera within the phyla Firmicutes representing >0.1% total abundance of fecal microbiota as identified by pyrosequencing. Circles represent independent variables and their proximity reflects their dissimilarity. The straight lines are dependent variables, and their lengths and angles between them are functions of the relative effects of independent variables.

7.5 DISCUSSION

Using a diet-controlled diet paradigm, the present study demonstrates, for the first time, that the USDA's recommended dose of ½ cup/d pulse crops facilitates shifts in the human gastrointestinal microbiome. Moreover, results reveal that different fractions of yellow peas, that vary in protein and fibre content, impose differential prebiotic effects.

Efficacy of pre- and probiotic dietary interventions are difficult to delineate in humans given the ability for background diets to modulate the gastrointestinal microbiome. Studies demonstrate that dietary records are an unreliable means of assessing whether food and energy intake remain stable throughout nutrition intervention studies (Schoeller, 1995). Given that colonic bacteria use food consumed by the host as a source of nutrition, the likelihood of observing type 1 or type 2 errors increases when assessing the effects of dietary interventions on the gastrointestinal microbiome when subjects are at liberty to alter their food and energy intake during active phases of clinical trials. Hence, studies, such as this one, that engage a highly controlled food and energy-intake paradigm where diet composition does not fluctuate during active phases represents an ideal study design for assessing pre-and probiotic interventions.

Typically, quantitative PCR and/or transcription fragment restriction length polymorphism analysis are utilized to identify changes in microbial populations subsequent to dietary interventions. Quantitative PCR is useful when investigators know which populations of microflora are expected to change with prebiotic interventions. While TRFL-P provides a more blanketed depiction of colonic bacterial abundance, it is a laborious and expensive technique. However, new technologies such as pyrosequencing allow researchers to, quickly and cost-effectively, obtain an overall depiction in abundance of the entire micro-genome at multiple taxonomic levels, from the phyla to genera, in response to dietary interventions. In addition, given the vast amount of data that are generated from pyrosequencing, studies that utilize pyrosequencing will identify

shifts in microbial populations that would otherwise not be explored if quantitative PCR and TRFL-P methodologies were engaged.

Although TRFL-P analysis revealed negligible effects of treatments on the microbial abundance at the level of phylum and class, pyrosequencing data were contradictory, noting significant treatment-induced shifts in the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria with WPF and FPF. The observation that Bacteroidetes and Firmicutes represented the most prominent phyla in the colonic microbiome is consistent with previous studies (Eckburg et al., 2005; Rajilic-Stojanovic et al., 2007; Suau et al., 1999). Moreover, modulation of bacterial abundance of Firmicutes and Bacteroidetes has been the focus of studies that investigate associations between gut microflora and metabolic disorders. For example, animal and human studies show that obesity is associated with a higher Firmicutes-to-Bacteroidetes ratio (Turnbaugh et al., 2008; Turnbaugh et al., 2009; Turnbaugh et al., 2006). In addition, inducing a change in phenotype from obese to lean via carbohydrate restricted or fat restricted diets, increased the proportion of Bacteroidetes relative to Firmicutes (Ley et al., 2006). Turnbaugh et al. (2006) suggest that, compared to Bacteroidetes, Firmicutes possess enhanced energy harvesting capabilities secondary to higher levels of genes that code for glycolytic enzymes. Given, that excess body weight has been associated with less desirable milieu of intestinal microflora (Backhed, 2009; Ley et al., 2005), overweight individuals utilized in the present study represent an ideal sample group to investigate prebiotic dietary interventions. Results from the present study demonstrate that both FPF and WPF increased levels of Bacteroidetes compared to WF in overweight and obese subjects. Moreover, despite concomitant increases in Firmicutes with WPF, FPF and WF, compared to WF, the ratio of Firmicutes-to-Bacteroidetes was 16 and 13% lower in WPF and FPF, respectively. Pyrosequencing analysis demonstrated favorable shifts in Bacteroidetes with FPF and WPF.

Higher proportions of Firmicutes have also been associated with good health in humans. A higher abundance and diversity of Firmicutes compared to Bacteroidetes was found in subjects without Crohn's disease compared to patients diagnosed with Crohn's Disease (Manichanh et al., 2006). Manichanh et al. (2006) suggest that a gastrointestinal microbiome with a high level of diversity confers the greatest health benefits to the host. Moreover, while discriminant analysis of phyla in the present study revealed considerable similarity between WPF and FPF, WPF and FPF demonstrated increased diversity of Firmicutes compared to WF (**Figure 13**). In addition, discriminant analysis of genera within Bacteroidetes and Firmicutes reveals greater bacterial diversity amongst FPF and WPF groups, respectively (**Figures 14 & 15**).

The most diverse phylum included in the pyrosequencing analysis was the Firmicutes. While WPF further enhanced the diversity of Eubacterium, Faecalibacterium, Ruminococcus, Sporobacter, and Phascolarctobacterium above WF and FPF, FPF enhanced Streptococcus, Coprococcus, Dorea and Roseburia compared to WPF and WF. It is unknown which perturbations in genera within Firmicutes from WPF and FPF elicit the greatest benefits to the host. Members of Firmicutes, as well as Bacteroidetes, are prominent short-chain fatty acid producers which are hypothesized to confer health benefits to the host by modulating insulin resistance, glucose metabolism, and lipid metabolism (Anderson & Bridges, 1984; Beauvieux et al., 2008; Robertson et al., 2005; Robertson et al., 2003). WPF and FPF impose similar, as well as distinct, effects on genera of the human microbiome.

Bifidobacterium and Lactobacillus have been shown to promote gastrointestinal health, local and systemic immunity and prevent metabolic endotoxemia (Boge et al., 2009; Cani et al., 2008; Cani et al., 2007; Ng et al., 2009). Despite Bifidobacterium and Lactobacillus being eliminated from pyrosequencing analysis since their relative abundance was <0.1% of the total microbiome, quantitative PCR demonstrated the WPF and FPF differentially favored the growth of Bifidobacterium and Lactobacillus,

respectively. Typically, the microbiome is substantially richer in Lactobacillus and Bifidobacterium during infancy compared to adulthood (Manson et al., 2008; Mariat et al., 2009). Given that Bifidobacterium and Lactobacillus represent the majority of microbiota utilized in the formulation of probiotic formulations, we postulate that dosages of WFP and FPF in the present study were too low to substantially increase the aforementioned genera of bacteria given the overwhelming populations of other microbes within the Firmicutes and Bacteroidetes categories. Nonetheless, we hypothesize that WPF and FPF could serve as functional ingredients that are added to probiotic formulations to serve as sources of nutrition for inoculative Lactobacillus and Bifidobacterium.

7.6 CONCLUSIONS

Results from the present study demonstrate that ½ cup/d yellow peas can favorably modulate the gastrointestinal microbiome in overweight and obese humans. In addition, the present study reveals that whole and fractionated pulse crops elicit different prebiotic effects given substantial differences in their macronutrient composition. Although the probiotic genera Lactobacillus and Bifidobacterium were increased with FPF and WPF, respectively, modulation of Lactobacillus and Bifidobacterium were minor when the entire microbiome was considered. Nonetheless, future studies will reveal whether WPF and FPF are effective nutrient-rich ingredients for probiotic bacteria in symbiotic formulations. The fact that whole and fractionated yellow peas elicited prebiotic effects when administered as flours lends further support to the concept that pulse-based flours are healthy functional ingredients.

7.4 ACKNOWLEDGMENTS

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CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

8.1 SUMMARY AND DISCUSSION

Whole yellow pea flour (WPF) and fractionated yellow pea flour (FPF) are novel functional food ingredients that can be utilized to increase pulse crop consumption, especially in North America. However, given that WPF and FPF vary in nutrient composition, the health benefits of WPF and FPF must be individually defined so that they can be confidently utilized as efficacious functional food ingredients that modulate risk factors linked to lifestyle associated diseases such as cardiovascular disease and diabetes. Results from the present research indicate that WPF and FPF impose similar as well as distinct benefits to health. Using a controlled dietary regimen that prevented weight fluctuations, consumption of ½ cup/d (50 g) WPF, and its equivalence in pea-derived fibre with FPF, by overweight men and women decreased fasting insulin and estimates of insulin resistance. Similar effects of WPF and FPF on insulin parameters indicate that the fibre fraction of yellow peas is responsible for their hypoinsulinemic and insulin-sensitizing effects. Moreover, WPF favored gynoid lipid deposition in women, a risk factor for insulin resistance. Compared to controls, FPF decreased post-prandial energy expenditure by lowering carbohydrate oxidation, while WPF maintained post-prandial substrate oxidation. Biscotti and banana bread formulated with WPF produced similar reductions in post-prandial glycemia when compared to boiled whole yellow peas, which were significantly lower than white bread. Golden Syrian hamsters consuming diets containing 10% FPF and WPF confirm the insulin-lowering properties of WPF and FPF. Moreover, results from the animal trial reveal that high doses of FPF can reduce fasting glucose levels, while WPF increases energy expenditure. Finally, WPF and FPF favorably modulate the human gastrointestinal microbiome by increasing the abundance and diversity of Firmicutes and Bacteroidetes. Results from the present research indicate that WPF and FPF are efficacious functional ingredients that impose similar as well as

distinct health benefits to humans within the realm of cardiovascular disease, diabetes and obesity.

The present research attempted to investigate the effects of WPF and FPF over a broad range of risk factors and morbidities associated with cardiovascular disease, diabetes and obesity. Although not successful in modulating all clinical endpoints investigated, parameters that were affected by WPF and FPF have clinical applications. Insulin resistance and hyperinsulinemia are risk factors for type II diabetes and cardiovascular disease (Rett, 1999). Furthermore, over 1/3 of American adults meet the criteria for diagnosis with Metabolic Syndrome (Ervin, 2009); a collection of comorbidities that includes insulin resistance (Cornier et al., 2008). Results from Chapter III indicate that FPF is a potent therapy for hyperinsulinemia and insulin resistance given that the human diet intervention utilized FPF at levels that were equivalent to the amount of pea-derived fibre in the WPF treatments. Moreover, 12 g/d FPF, containing 10 g/d pea derived fibre, is considerably easier to incorporate into human diets compared 53 g/d WPF. Visceral adiposity is also a risk factor for insulin resistance as well cardiovascular disease and atherosclerosis (Despres, 1993). Results from Chapter III demonstrate that WPF decreases android adiposity, which is mostly visceral lipid, in women. In addition, levels of enterolactone tended to be higher in subjects consuming WPF treatments. Although the present data cannot confirm why lipid deposition was only demonstrated in women, we hypothesize an interaction between pea-derived phytoestrogens and estrogen receptor α, which are more prevalent in the subcutaneous adipose tissue of females (Richelsen, 1986). Overall, WPF and FPF possess hypoinsulinemic properties.

Typically, humans consume yellow peas as whole or split peas after boiling and/or soaking. Previous studies suggest that the physical structure of whole pulse crops could be responsible for their low post-prandial glycemic properties. For example, given their small particle size, researchers hypothesize that flours are hyperglycemic compared to whole foods because flours possess higher surface area exposure to heat and water during cooking, and to glycolytic enzymes during digestion in the small intestine (Bjorck et al.,

1994; Golay et al., 1986; Tovar et al., 1992). However, results from the dietary interventions utilized in Chapter IV demonstrate that banana bread and biscotti formulated with WPF are low glycemic foods and could be used by type II diabetics to manage post-prandial glucose levels.

Certain patient populations serve to benefit from ingredients such as FPF that lower thermogenesis. For instance, individuals suffering from chronic obstructive pulmonary disease (COPD) struggle to digest carbohydrate because of carbohydrate's high respiratory quotient. Consequently COPD patients are often advised to consume higher fat diets (Cai et al., 2003). Thus, adding FPF to meals might help maintain the carbohydrate content of diets amongst COPD patients by reducing and/or slowing post-prandial carbohydrate oxidation. WPF's failure to enhance thermogenesis in the human intervention described in Chapter V is likely secondary to the low dose of WPF utilized relative to the high caloric density of the background diet that maintained body weight amongst subjects over the duration of active study phases. Conversely, results from Chapter VI demonstrated that hamsters consuming high doses of WPF increased post-prandial energy expenditure and suggest that incorporation of WPF into diets, at levels greater than USDA recommended dosages, facilitates thermogenesis. Overall the effects of WPF and FPF on post-prandial thermogenesis and substrate utilization require further investigation.

The observation that WPF and FPF were able to modulate the abundance and diversity of Firmicutes and Bacteroidetes in humans and hamsters could lend further support to whole and fractionated pulse crops as functional food ingredients. Firmicutes and Bacteroidetes include bacterial genera that degrade fibre and produce bioactive molecules, such as short-chain fatty acids, that impose local as well as systemic benefits to the host (Anderson & Bridges, 1984; Robertson, 2007; Thorburn et al., 1993). In addition, quantitative PCR analysis revealed that FPF and WPF selectively increased Lactobacillus and Bifidobacterium, respectively, two common probiotic genera associated with gastrointestinal health (Lomax & Calder, 2009b). Results suggest that FPF and WPF can

be utilized as prebiotic dietary ingredients or constituents of symbiotic dietary supplements.

The most controversial effect of pulse crop consumption on human health is their ability to modulate circulating lipid levels. Previous literature provides evidence supporting and refuting pulse crops' lipid-lowering efficacy. In studies demonstrating that pulse crops modulate lipid concentrations, fibre is usually identified as the bioactive component. Fibres have been shown to modulate hepatic lipid metabolism and/or bind dietary lipid and bile acids in the intestine, facilitating their excretion (Elhardallou, 1992). Nonetheless, results from Chapter III and VI demonstrate that USDA recommended dosages of WPF and FPF in humans and high fat diets containing 10% WPF and FPF in hamsters failed to decrease circulating cholesterol and triglyceride levels. Results suggest that yellow peas' ability to reduce lipids are secondary to other mechanisms of action besides a direct of effect of fibres on lipid metabolism.

8.2 FUTURE DIRECTIONS

Although results from the present research provide important insight into the efficacy of WPF and FPF as functional dietary ingredients, further exploration within certain investigative themes are necessary. First, the human clinical trials described in Chapter III and V examined post-prandial responses of WPF and FPF when incorporated into a mixed meal. However, the direct effects of FPF and WPF on post-prandial energy expenditure, substrate oxidation, hepatic lipid/glucose metabolism and glucose levels should be explored without interference from background diets. Investigating the exclusive metabolic effects of yellow pea-derived flours would give further insight regarding the best approach to capitalize on the health benefits of WPF and FPF. Second, dose-response studies in humans will decipher optimal levels of WPF and FPF required to modulate specific clinical endpoints. Third, studies must determine if WPF and FPF are effective at influencing clinical endpoints outside the realm of a diet-controlled intervention study. Such studies could also reveal indirect benefits of whole and

fractionated pea flours on clinical endpoints such as lipids via fibre-induced satiety. Finally, further exploration into the prebiotic effects of WPF and FPF is warranted. As described throughout the present body of research, fibres facilitate the growth and proliferation of "good" colonic bacteria and impose local and systemic benefits to the host. Correlative and bioinformatic analysis between shifts in gastrointestinal microbial populations and clinical endpoints could unlock additional benefits of WPF and FPF consumption. Exploration of WPF and FPF consumption within the context of the aforementioned themes will bring further insights into how pulse crops prevent and help manage cardiovascular disease, diabetes and obesity.

8.3 FINAL CONCLUSIONS

Whole yellow peas represent Canada's most prevalent pulse crop commodity. However, over 80% of Canadian yellow peas are exported to other countries for human consumption. Similar to other pulse crops, yellow peas are high in protein, fibre and antioxidants while low in fat. The present body of research has identified that whole and fractionated yellow peas modulate risk factors and comorbidities associated with cardiovascular disease, diabetes and obesity. In addition, different components of yellow peas produce beneficial shifts in the colonic microbiome. By elucidating the effects of yellow pea consumption on health outcomes, yellows peas will have enhanced value as a commodity and, with anticipation, facilitate greater retention of Canadian yellow peas within North America for human consumption. Results from the present series of studies represent meaningful advances in unraveling the health benefits of consuming whole and fractionated pulse crops as novel functional food ingredients.

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APPENDICES

APPENDIX I

ETHICS APPROVAL FOR STUDIES CORRESPONDING TO CHAPTERS III, V AND VII

SEP 2 5 2006

UNIVERSITY of MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: Pulse Canada

Protocol Reference Number: B2006:129
Date of REB Meeting: August 28, 2006
Date of Approval: September 15, 2006
Date of Expiry: August 28, 2007

Protocol Title: "Effect of Pulses and Pulse Fractions on Indices of Lipid, Carbohydrate and Energy Metabolism, as well as Oxidative Status in Overweight, Hyperlipidemic Individuals"

The following is/are approved for use:

- **Protocol dated August 2006**
- **Research Participant Information and Consent Form dated September 15, 2006**
- **Advertisement submitted August 14, 2006**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letter dated September 8, 2006 and electronic mail dated September 18, 2006. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations*.

This approval is valid for one year from the date of the meeting at which it was reviewed. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

COPY

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255/ **Fax:** (204) 789-3414

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Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: Pulse Canada

Protocol Reference Number: B2006:129
Date of Approval: December 19, 2006

Protocol Title: **"Effect of Pulses and Pulse Fractions on Indices of Lipid, Carbohydrate and Energy Metabolism, as well as Oxidative Status in Overweight, Hyperlipidemic Individuals"**

The following is/are approved for use:

- **Protocol amendment submitted December 15, 2006**
- **Research Participant Information and Consent Form dated December 15, 2006**
- **Newspaper advertisement submitted December 15, 2006**
- **Online Advertisement submitted December 15, 2006**
- **General Information Sheet submitted December 15, 2006**
- **MTS Centre Advertisement submitted December 15, 2006**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee as per your letter dated December 15, 2006. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations.*

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Sincerely yours,

JAN 03 2007

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255/ **Fax:** (204) 789-3414

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BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

FEB 15 2007

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: Pulse Canada

Protocol Reference Number: B2006:129
Date of Approval: February 8, 2007

Protocol Title: **"Effect of Pulses and Pulse Fractions on Indices of Lipid, Carbohydrate and Energy Metabolism, as well as Oxidative Status in Overweight, Hyperlipidemic Individuals"**

The following is/are approved for use:

- **Advertisements submitted February 7, 2007**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee as per your facsimile dated February 7, 2007. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations*.

A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255/ **Fax:** (204) 789-3414

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: Pulse Canada

Protocol Reference Number: B2006:129
Date of Approval: February 26, 2007

Protocol Title: **"Effect of Pulses and Pulse Fractions on Indices of Lipid, Carbohydrate and Energy Metabolism, as well as Oxidative Status in Overweight, Hyperlipidemic Individuals"**

The following is/are approved for use:

- **Amendment per letter dated February 20, 2007**
- **Research Participant Information and Consent Form, Version dated February 20, 2007**
- **Revised Advertisement submitted February 20, 2007**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee as per your letter dated February 20, 2007. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations.*

A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255/ **Fax:** (204) 789-3414

Appendix I: Ethics approval corresponding to chapters III, V & VII

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: Pulse Canada

Ethics Reference Number: B2006:129
Date of Approval: August 15, 2008
Date of Expiry: August 28, 2009

Protocol Title: "Effect of Pulses and Pulse Fractions on Indices of Lipid, Carbohydrate and Energy Metabolism, as well as Oxidative Status in Overweight, Hyperlipidemic Individuals"

The following is/are approved for use:

- **Annual Approval**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, and University of Manitoba on behalf of the committee per letter dated August 12, 2008. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations*.

This approval is valid until the expiry date only. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255/ **Fax:** (204) 789-3414

APPENDIX II

ETHICS APPROVAL CORRESPONDING TO CHAPTER IV

Mondei - for file

FEB 1 4 2008

UNIVERSITY OF MANITOBA

BANNATYNE CAMPUS
Research Ethics Boards

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. P. Jones
Sponsor: MFFOP

Protocol Reference Number: B2008:017
Date of REB Meeting: January 28, 2008
Date of Approval: February 13, 2008
Date of Expiry: January 28, 2009

Protocol Title: "Palatability and acute glycemic response to pea flour baked foods"

The following is/are approved for use:

- **Protocol dated February 6, 2008**
- **Research Participant Information and Consent Form, Version dated February 6, 2008**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letter dated February 5, 2008 and electronic mail dated February 12, 2008. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations.*

This approval is valid for one year from the date of the meeting at which it was reviewed. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

NR Anthonisen

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above protocol reference number on all correspondence.
Inquiries should be directed to the REB Secretary
Telephone: (204) 789-3255/ **Fax:** (204) 789-3414

APPENDIX III

ETHICS APPROVAL CORRESPONDING TO CHAPTER VI

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

Room 106
196 Innovation Drive
Winnipeg, Manitoba
Canada R3T 2N2
Telephone (204) 474-9787
Fax (204) 474-7552
peter_jones@umanitoba.ca

To: Protocol Management and Review Committees - Fort Garry Campus
C/O Tracy Van Osch

From: Dr. Peter Jones

Re: Renewal of Animal Care Protocol #F06-013

Please find attached the renewal - short form for protocol #F06-013 newly titled "Effect of functional plant and algal products on cholesterol and fatty acid kinetics." The only changes to the entire protocol are the composition of the test diets. All added components are safe food grade material which will be added at functional levels to determine efficacy of affecting changes to lipid kinetics and body composition. The attached table 1 outlines the composition of the basic control diet which is designed to meet all nutritional requirements while inducing atherosclerosis and obesity in these hamsters. The test articles are added as a percentage by weight of the diet at the expense of corn starch.

Test Articles and dose (percentage by weight):

Control - no test article
Plant sterol control - 2%
Wheat bran - 10%
Novel treated wheat bran - 10%
Yellow pea flour (no hull) - 10%
Yellow pea flour (with hull) - 10%
Green hypertrophic freshwater algae biomass - 10%
Green hypertrophic freshwater algae biomass - 5%

Please feel free to contact myself or Dr. Scott Harding (474-7842) with any questions or concerns.

UNIVERSITY OF MANITOBA | OFFICE OF RESEARCH SERVICES
Office of the Vice-President (Research)

CTC Building
208 - 194 Dafoe Road
Winnipeg, MB R3T 2N2
Fax (204) 269-7173
www.umanitoba.ca/research

July 24, 2007

TO: Dr. P. Jones, Richardson Centre for Functional Foods & Nutraceuticals, 196 Innovation Drive

FROM: Dr. T. Dick, Chair, Fort Garry Campus Protocol Management and Review Committee

RE: **Renewal of Protocol F06-013**

Please be advised that your request for renewal of your project entitled **"Effect of algal polysaccharides on indices of health status in hamsters."** (category of invasiveness "B"), has been approved for the period ending **April 30, 2008. The reference number for this protocol is now F06-013/1.** The protocol reference number must be used when ordering animals.

It is understood that these animals will be used only as described in your protocol. The protocol must be kept current. Should changes become necessary, very minor alterations can be made with the approval of the University Veterinarian, provided that the protocol in Central files is amended appropriately. More substantive changes will require resubmission to and reassessment by the Bannatyne Campus Protocol Management and Review Committee. If approved, this will result in the assignment of a new protocol reference number.

Failure to follow this protocol, or renew it prior to the expiry date, will result in the termination of your ability to continue using or ordering animals. **Please be advised that only three renewals are allowed. Subsequently, a full application must be submitted.**

TD/lh

c.c. Veterinary Services
Ms. T. Whittington, RCFFN Animal Holding Facility

APPENDIX IV

FORMS CORRESPONDING TO STUDIES DESCRIBED IN CHAPTERS III, V & VII

CONSENT FORM

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study: **Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.**

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-8883

Co-Investigator: Trust Beta, PhD
Department of Food Science
University of Manitoba
Winnipeg, MB R3T 6C5
Phone: (204) 474-8214

Linda Malcolmson, PhD
Canadian International Grains Institute
1000-303 Main Street
Winnipeg, Manitoba, R3C 3G7
Phone: (204) 983-8548

Curtis Rempel, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-6030

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

 Initials of Subject: _____

Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.

Purpose of Study

This clinical trial is being conducted to study the safety and efficacy of pulse (bean, lentil, pea and chickpea) consumption on blood cholesterol levels as well as parameters related to diabetes, obesity and cancer. You are being asked to take part in this study because you are a healthy man or woman, aged 18 – 60 yr with elevated cholesterol levels and are overweight or slightly obese.

A total of 30 participants will be participating in the study.

Study Procedures

If you agree to take part in this study, as part of a pre-screening visit, you will be asked to have a fasting (nothing to eat or drink 12 hours before the test) blood sample of approximately 15 – 20 ml of blood will be drawn to measure your blood lipid levels. If you meet eligibility requirements, you will be invited back for further screening where a fasting blood sample of 15 – 20 ml will be drawn in order to conduct a complete blood count, blood sugar, electrolytes, and a complete lipoprotein analysis (triglyceride, total, very low density lipoprotein (VLDL) cholesterol, high density lipoprotein (HDL) cholesterol and LDL cholesterol). Prior to study commencement, you will also undergo a physical examination by a physician to ensure that you are in good health. An electrocardiogram (EKG) may be performed at the discretion of the physician in charge at this time.

You will be ineligible to participate in this clinical research study if you: (i) have recently used (i.e. within the last 3 months) or chronically used oral hypolipidemic therapy, including fish oils or probucol; (ii) habitually use alcohol (> 2 drinks / day); (iii) have had a heart attack, coronary artery bypass, or other major surgical procedures within the last six months; (iv) have recent onset of angina, congestive heart failure, inflammatory bowel disease, diabetes, or hypothyroidism; (v) have cancer (evidence of active lesions, chemotherapy or surgery in the past year); (vi) are a continuous user of fiber laxative (greater than 2 doses / week), or stimulant laxatives (vii) have a history of eating disorders; (viii) exercise greater than 15 miles / week or 4,000 kcal / week.

This study will be a cross-over study consisting of three controlled dietary phases. Each phase will be four weeks in duration. Each controlled dietary phase will be separated by a 4 week washout phase. During the 4 week controlled dietary phase you will be required to come to the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) daily, including weekends, for at least one meal per day. During the 4 week washout phase, you will not be required to come to the RCFFN on a daily basis, and you will consume your habitual diet.

During each treatment period trial subjects will consume prepared nutritionally adequate solid food diets as three meals per day, prepared at the Richardson Clinical Nutrition Research Unit (RCNRU), where at least one meal per day will be consumed under supervision. The remaining

 Initials of Subject: _____

Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.

meals will be prepared and packaged for take out and you will be allowed to take them home with you. Diets will be consistent in composition across each 4 week period, designed using nutrient composition databases, to contain 35% energy as fat, 50% carbohydrate and 15% protein. Pulse will be integrated to bagels / muffins / snack bars / pasta which will comprise a part of the meals at breakfast and supper.

As a study participant, you will receive the following dietary treatments in each of the three phases:

- Phase I: Control phase with regular bagels / muffins / snack bars / pasta;
- Phase II: Pulse phase where bagels / muffins / snack bars are provided containing the equivalent of 3 cups of pulses / week;
- Phase III: Pulse fractions phase where bagels / muffins / snack bars / pasta are provided containing the equivalent of 3 cups of pulses / week.

At the beginning and end of each of the three treatment phases (a total of six times), you will have your body fat measured using a procedure called dual energy x-ray absorptiometry (DEXA). For the DEXA procedure, you will need to lie in a horizontal position on a padded table for approximately 5 minutes while the scanning arm of the DEXA machine passes from your head to your feet. The radiation dosage from a DEXA scan is extremely low – it is equivalent to approximately 1 day of exposure to natural background radiation. This dosage is 1000 times less than the limit for trivial exposure. You are requested not to wear any metal object during this test as metal may affect the bone density values, which in turn affect your body composition calculations and lead to erroneous data.

During days 1, 2, 27 and 28 of each four-week test diet phase, fasting blood samples will be drawn for assessment of various biological markers including blood fat and sugar levels. Drawing of each blood sample will take approximately 3 minutes. Approximately 15 – 20 ml of blood will be draw at each sampling date for a maximum of 280 ml for the entire study. Urine samples will also be provided on these same days.

In addition, you will have the option of provide fecal samples at the end of each treatment phase. You will be provided the appropriate apparatus for fecal collection in your home. Further financial remuneration will be implemented if you choose this optional treatment arm of the study.

On the second to last day of each four-week test diet phase, you will be asked to drink approximately three tablespoons of water containing deuterium (a non-radioactive substance, also commonly called heavy water) in order to establish the amount of triglyceride being produced inside your body. Heavy water is almost identical to regular water (H_2O), except that a heavier form of hydrogen called deuterium replaces the hydrogen part of the water molecule. This substance is non-radioactive and poses no toxicity hazard.

 Initials of Subject: _____

Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.

On the night before the last day of each four-week phase of the study, you will be asked to fast starting at 8 pm in order for the next day's test which will include eating a piece of bread with margarine that contains a non-radioactive tracer in order to establish how fats are used in the body. This test will require breath samples. This will involve breathing into a special plastic bag dedicated to breath sample collection. These breath samples will be taken every hour for 6 hours following consumption of the non-radioactive tracer and again after 24 hours. The breath sampling can be performed either at the Richardson Centre for Functional Foods and Nutraceuticals or a small kit to collect breath samples will be available for you to take home and collect yourself. During the first 6 hours of breath sample collection you will be asked to refrain from eating or drinking anything besides water.

During the first and fourth week of each dietary phase, you will have your energy expenditure measured using a canopy hood ventilation system. You will have a plexiglass canopy put over your head for 30 minutes before breakfast to obtain the first measurement, and 5.5 hours after breakfast to obtain the second measurement. You will be asked to consume breakfast within 30 minutes after the first measurement and before the second measurement. You can breathe normally during the test because the air in the room is directed through the hood. The rate at which your body is burning calories will be determined by measuring the rate of oxygen that you consume and the carbon dioxide that you produce while the ventilation system is in operation.

Risks and Discomforts

Pulses, commonly defined as 'the edible seeds of leguminous plants cultivated for food, as peas, beans, lentils and chickpeas, etc.,' are excellent food choices because of their health promoting benefits. Important components include vegetable protein, complex carbohydrate, dietary fibre, vitamins, mineral and other components. The dietary treatment of 3 cups of pulses per week follows recommendation from the USDA Dietary Guideline (2005) for legume consumption.

The pulse or pulse fractions contained within the meals at the proposed level has been shown to have no known direct negative side effects on health. Pulses are a dietary staple in many regions of the world. All of the pulse flours or fractions are commercially available in supermarkets, grocery and health food stores.

One of the noted adverse effects of a diet high in pulses is flatulence, due to the oligosaccharide content present in pulse seeds. However, the digestive tract becomes adapted to the increased oligosaccharide content in a short timeframe.

Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site.

Initials of Subject: _____

Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.

Benefits

By participating in this study, you will be providing information to the study doctors that will show the effects of pulses and pulse fractions in diets on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals. There may or may not be direct medical benefit to you from participating in this study.

Costs

All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Payment for Participation

You will receive compensation for your time and inconvenience of the study schedule. You will be given $333.33 upon completion of each four-week study phase up to a maximum total compensation of $1000.00 If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

If you choose the option of providing a fecal sample at the end of each treatment phase, an additional $200.00 will be provided. In the end, you will receive $400.00 upon completion of each four-week study phase up to a maximum total compensation of $1200.00. If you withdraw early form the study, you will receive an appropriate pro-rated fraction amount dependent on whether you provided a fecal sample.

Alternatives

You do not have to participate in this study. The study doctor will answer any questions you have about the experimental group of this study.

Confidentiality

Information obtained in this research study may be published or presented in public forums. However your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The Richardson Centre for Functional Foods and Nutraceuticals staff involved with your care may review/copy medical information that may reveal your identity to them. All records will be kept in a locked secure area. No information revealing any personal information such as your name, address or telephone number will leave the Richardson Centre for Functional Foods and Nutraceuticals.

 Initials of Subject: _____

Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.

With your permission, the study doctor may also write to your Family Doctor to tell him/her that you are taking part in a study or to obtain further medical information.

The Biomedical Research Ethics Board at the University of Manitoba may also review your research-related records for quality assurance purposes. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the Richardson Centre for Functional Foods and Nutraceuticals.

Study samples will be stored in the freezer at the Richardson Centre for Functional Foods and Nutraceuticals. Only the study coordinators and the principal investigator will have access to the samples. Your samples will not be used for any additional analyses, will not be stored for any longer than 2 years, nor shared with any other group, other than is indicated in the protocol, without your specific consent.

Voluntary Participation/Withdrawal From the Study

Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

Your participation in this study may be terminated without your consent by the study doctor. Your study doctor will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study doctor.

If you decide to participate, you agree to cooperate fully with the study visit schedule, and will follow the study doctor's instructions.
We will inform you about new information that may affect your health, welfare, or willingness to stay in this study.

Medical Care for Injury Related to the Study

In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Kesselman at (204) 954-4486 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Kesselman will be contacted and will inform you of the results.

 Initials of Subject: _____

Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

Investigator:	Dr. Peter Jones	Tel No.	204-474-9787
Study Physician	Dr. Edward Kesselman	Tel No.	204-954-4486

For questions about your rights as a research subject, you may contact:
The Biomedical Research Ethics Board, University of Manitoba at 789-3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Initials of Subject: _____

Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with Dr. Peter Jones and/or his study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to participate in the research study by any statement or implied statements. Any relationship (such as employee, student or family member) I may have with the study team has not affected my decision to participate. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my medical records by Richardson Centre for Functional Foods and Nutraceuticals, the Health Protection Branch, and The University of Manitoba Biomedical Research Ethics Board.

By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

I would agree to provide a fecal sample at the end of each treatment phase.

Yes ☐ No ☐ Initials: _____

I agree to being contacted in relation to this study.

Yes ☐ No ☐

I agree to my family physician being notified of my participation in this study if required.

Yes ☐ No ☐

Participant signature: _____

Participant printed name: _____

Date _____
(Day/month/year)

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given his or her consent

Initials of Subject: _____

Appendix IV: Patient consent form

Effect of pulses and pulse fractions on indices of lipid, carbohydrate and energy metabolism, as well as oxidative status in overweight, hyperlipidemic individuals.

Printed Name: _____

Signature: _____

Date _____
(day/month/year)

Role in the study: _____

Initials of Subject: _____

Appendix IV

PATIENT SCREENING FORM

Pulse Canada 2007 Screening Form

DATE (yyyy/mm/dd) _____/_____/_____

Personal Information

Name:

Sex:

☐ Male ☐ Female

Telephone:
(Home) ( ) (Work): ( ) (Cell): ( )

Age (18-60):
_____

DOB (yyyy/mm/dd):
_____/_____/_____

Weight:
_____lbs _____kg

1kg = 2.2 lbs

Height:
____ft ____in _____cm _____m

1 inch = 2.54 cm

BMI **(office use only)**:
_____ kg/m^2

Smoker:

☐ YES ☐ NO

Physical Activity

☐ YES ☐ NO

If yes, 15 miles/week or 4000 kcal/wk

☐ YES ☐ NO

Appendix IV: Patient Screening form

Medications/Supplements

Prescription Medication or natural health product?	☐ YES ☐ NO
Cholesterol-lowering Medications Past 6 mo?	☐ YES ☐ NO
If yes, please specify:	☐ Cholestyramine ☐ Colestipol ☐ Niacin ☐ Gemfibrozil ☐ Probucol ☐ HMG-CoA reductase inhibitors ☐ Guggul ☐ Lecithin ☐ Evening primrose oil ☐ Other _____

Cholesterol-lowering medications or products cannot be consumed during the study

Thyroid Medication:	☐ YES ☐ NO
If yes, are your doses stable?	☐ YES ☐ NO
Blood pressure medication?	☐ YES ☐ NO
Weight Loss Supplement or product (Natural or Pharmacological)	☐ YES ☐ NO
Fish Oil Capsules in the last 6 mo (> 4 g/day)?	☐ YES ☐ NO
Supplements containing plant sterols or phytosterols within the last 6 weeks?	☐ YES ☐ NO
Herbal or Food Supplements	☐ YES ☐ NO If yes, please specify: _____ _____ dose: _____ Is this dose stable ☐ YES ☐ NO
Vitamin and Mineral Supplments	☐ YES ☐ NO If yes, please specify: _____

Appendix IV: Patient Screening form

_____ dose: _____

Is this dose stable ☐ YES ☐ NO

Medical History

Have you experienced the following during the past 3 months?

☐ Diabetes mellitus
☐ Thyroid
☐ Kidney disease
☐ Liver disease
☐ Heart disease

Metallic Bone Components?

☐ YES ☐ NO

Dietary Habits

Special dietary habits?

i.e. vegetarian

☐ YES ☐ NO

If yes, please specify: _____

_____

Food allergies?

Yellow peas, eggs, milk (lactose) etc

☐ YES ☐ NO

If yes, please specify: _____

_____

Specific Likes and Dislikes	**LIKES**	**DISLIKES**

Do you have a history of alcohol consumption(> 2 glasses/ (every) day)?

☐ YES ☐ NO

Appendix IV: Patient Screening form

ELIGIBLE FOR BLOOD SCREENING (office use only)
☐ YES ☐ NO

Blood and Medical Screening Appointments (office use only)

First Blood Screening (lipids)

Date (yyyy/mm/dd): _____/_____/_____

Time: ___:___

Second Blood Screening and Medical Exam

Second Blood Screening (lipids and biochemistry)	**Medical Exam**
Second: Date (yyyy/mm/dd): _____/_____/_____ Time: ___:___	First Date (yyyy/mm/dd): _____/_____/_____ Time: ___:___
	Second (at physicians request): Date (yyyy/mm/dd): _____/_____/_____ Time: ___:___

ELIGIBLE FOR STUDY(office use only)
☐ YES ☐ NO

Start date (yyyy/mm/dd):

_____/_____/_____

Subject Code:

_____

Appendix IV

MEDICAL SCREENING FORM

Pulse 2007
Screening Medical Examination Form

Phase Screening	**Study Physician** Dr. Edward Kesselman	**Subject Code**
Date of Visit _____/_____/_____ MM DD YR	**Investigator** Dr. Peter Jones	

COMPLETE PHYSICAL EXAMINATION

A. Vital Signs

Body Weight:_____ lbs _____ kg **Height:** _____cm

Respiration:_____

Blood Pressure (seated): _____ / _____ mmHg **Heart Rate:** _____ bpm
systolic diastolic

Race/Ethnic Origin:
☐ **Caucasian** ☐ **African-American/Canadian** ☐ **Asian** ☐ **Other:**_____

B. Body Systems (Check the appropriate box if organ system was examined. If not done, write N/D in the box)

	Normal	Abnormal	*Details of abnormal finding
1) Ears, Nose, Throat			
2) Eyes			
3) Dermatological			
4) Musculoskeletal			
5) Lymph Nodes			
6) Neurological			
7) Cardiovascular 8) Respiratory			
9) Endocrine			
10) Urogenital			
11) Gastrointestinal (complete section C)			

C. Gastrointestinal Cont…

Bowel Habits: Frequency_____/Day **Urination:** Frequency_____/Day

Consistency_____ Nocturia_____/Night

Medications:

Pulse 2007
Screening Medical Examination Form

Hospitalizations: Family History:

D. Medical History Exclusion Criteria Screening Questionnaire		
	YES	**NO**
Have you taken a medication affecting lipid metabolism (cholestyramine, colestipol, niacin, colfibrate, gemfibrozil, probucol, HMG-CoA reductase inhibitors, and high-dose dietary supplements, plant sterols or fish oil capsules) within the past 3 months?		
Do you take any natural or pharmaceutical weight loss supplements or products?		
Do you smoke?		
Do you consume large amounts of alcohol? (more than 2 drinks per day or 12 drinks per week)		
Do you have diabetes mellitus?		
Do you have kidney disease?		
Do you have liver disease?		
Do you have heart disease?		
Do you have uncontrolled thyroid disease or hypertension? (Subject will be accepted if she is on a stable dose of a thyroid or blood pressure medication that has no known effects on blood lipid metabolism.)		
Are you pregnant or do you intend to become pregnant?		

Pulse 2007
Screening Medical Examination Form

E. Additional Physician Notes
Based on the inclusion and exclusion criteria above, and the medical exam is the subject eligible to participate in the study protocol (circle one): **YES** **NO**
Physician's Signature:_____ Date:_____

Appendix IV

STUDY PROGRESS FORM

Patient Study Progress

Study ID: _____

1. Day 1 (mm/dd/yyyy): ___/___/___ ☐

- DEXA
- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection

2. Day 2 (mm/dd/yyyy): ___/___/___ ☐

- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection

3. Week 1 (mm/dd/yyyy): ___/___/___ ☐

- Energy Expenditure
- Glucose monitoring

4. Week 1 Side Effects Questionnaire: ___/___/___ ☐

---

5. Day 28 (mm/dd/yyyy): ___/___/___ ☐

- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection
- $^{2}H_2O$ administration (before blood) **Time:** ____: ____
- Side Effects Questionnaire

6. Day 29 (mm/dd/yyyy): ___/___/___ ☐

- DEXA before breakfast
- Blood 2 EDTA, 2 Serum, 2 Heparin **Time:** ____: ____
- Urine Collection

7. Week 4 (mm/dd/yyyy): ___/___/___ ☐

- ^{13}C administration (with meal)
- Energy Expenditure
- Breath sampling (before and after breakfast)
- Glucose monitoring (before and after meal)

8. Week 4 Side Effects Questionnaire: ___/___/___ ☐

PHASE 2

9. Day 56 (mm/dd/yyyy): ___/___/___ ☐

- DEXA
- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection

10. Day 57 (mm/dd/yyyy): ___/___/___ ☐

- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection

11. Week 1 (mm/dd/yyyy): ___/___/___ ☐

- Energy Expenditure
- Glucose monitoring

12. Week 1 Side Effects Questionnaire: ___/___/___ ☐

---

13. Day 84 (mm/dd/yyyy): ___/___/___ ☐

- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection
- $^{2}H_2O$ administration (before blood) **Time:** ____: ____
- Side Effects Questionnaire

14. Day 85 (mm/dd/yyyy): ___/___/___ ☐

- DEXA before breakfast
- Blood 2 EDTA, 2 Serum, 2 Heparin **Time:** ____: ____
- Urine Collection

15. Week 4 (mm/dd/yyyy): ___/___/___ ☐

- ^{13}C administration (with meal)
- Energy Expenditure
- Breath sampling (before and after breakfast)
- Glucose monitoring (before and after meal)

16. Week 4 Side Effects Questionnaire: ___/___/___ ☐

PHASE 3

17. Day 112 (mm/dd/yyyy): ___/___/___ ☐

- DEXA
- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection

18. Day 113 (mm/dd/yyyy): ___/___/___ ☐

- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection

19. Week 1 (mm/dd/yyyy): ___/___/___ ☐

- Energy Expenditure
- Glucose monitoring

20. Week 1 Side Effects Questionnaire: ___/___/___ ☐

---

21. Day 140 (mm/dd/yyyy): ___/___/___ ☐

- Blood 2 EDTA, 2 Serum, 2 Heparin
- Urine Collection
- $^{2}H_2O$ administration (before blood) **Time**: ____: ____
- Side Effects Questionnaire

22. Day 141 (mm/dd/yyyy): ___/___/___ ☐

- DEXA before breakfast
- Blood 2 EDTA, 2 Serum, 2 Heparin **Time:** ____: ____
- Urine Collection

23. Week 4 (mm/dd/yyyy): ___/___/___ ☐

- ^{13}C administration (with meal)
- Energy Expenditure
- Breath sampling (before and after breakfast)
- Glucose monitoring (before and after meal)

24. Week 4 Side Effects Questionnaire: ___/___/___ ☐

Appendix IV

PATIENT MENSTRUAL CYCLE CHECKLIST

MENSTRUAL CYCLE CHECKLIST

PHASE 1

-Start date (day 1) (dd/mm/yyyy) ___/___/___

-Day 1 of last cycle (dd/mm/yyyy) ___/___/___

-Calculated Day 14 of cycle (dd/mm/yyyy) ___/___/___

-Pregnancy Test __ **Yes** __ **No**

-Results __ **Positive** __ **Negative**

PHASE 2

-Start date (day 1) (dd/mm/yyyy) ___/___/___

-Day 1 of last cycle (dd/mm/yyyy) ___/___/___

-Calculated Day 14 of cycle (dd/mm/yyyy) ___/___/___

-Pregnancy Test Results __ **Yes** __ **No**

-Results __ **Positive** __ **Negative**

PHASE 3

-Start date (day 1) (dd/mm/yyyy) ___/___/___

-Day 1 of last cycle (dd/mm/yyyy) ___/___/___

-Calculated Day 14 of cycle (dd/mm/yyyy) ___/___/___

-Pregnancy Test Results __ **Yes** __ **No**

-Results __ **Positive** __ **Negative**

Appendix IV

PATIENT FECAL COLLECTION PROGRESS FORM

FECAL OPTION CHECKED ON CONSENT

☐ YES ☐ NO

PHASE 1

1. Wednesday (dd/mm/yyyy) ___/___/____ ☐
2. Friday (dd/mm/yyyy) ___/___/____ ☐

PHASE 2

1. Wednesday (dd/mm/yyyy) ___/___/____ ☐
2. Friday (dd/mm/yyyy) ___/___/____ ☐

PHASE 3

1. Wednesday (dd/mm/yyyy) ___/___/____ ☐
2. Friday (dd/mm/yyyy) ___/___/____ ☐

Appendix IV

PATIENT SIDE EFFECTS FORM

Pulse Study 2007 Treatment Side-effects Form

Study ID#: _____ **Study Phase: _____**

Date (mm/dd/yyyy): _____/_____/_____

1. How long have you been receiving your current treatment?

☐ Approximately 1 week (7 days)
☐ Approximately 4 weeks (28 days)

3.How many 28 day treatment phases have completed prior to this one?

☐ 0 ☐ 1 ☐ 2

2. Have you experienced any gastrointestinal side-effects since initiating this particular treatment?

☐ Diarrhea
☐ 1 - 2 days ☐ 3 - 5 days ☐ 1 week ☐ > 1 week

If greater than 1 week, how long did you experience diarrhea? _____

Has this symptom resolve itself? ☐ Yes ☐ No

Did you initiate pharmacological treatment for diarrhea ☐ Yes:_____ ☐ No

---

☐ Cramping
☐ 1 - 2 days ☐ 3 - 5 days ☐ 1 week ☐ > 1 week

If greater than 1 week, how long did you experience cramping? _____

Has this symptom resolve itself? ☐ Yes ☐ No

Did you initiate pharmacological treatment for cramping? ☐ Yes:_____ ☐ No

---

☐ Increased Flatulence
☐ 1 - 2 days ☐ 3 - 5 days ☐ 1 week ☐ > 1 week

If greater than 1 week, how long did you experience increased flatulence? _____

Has this symptom resolve itself? ☐ Yes ☐ No

Did you initiate pharmacological treatment for increased flatulence?
☐ Yes:_____ ☐ No

Modified March 30, 2007

☐ Nausea

☐ 1 - 2 days ☐ 3 - 5 days ☐ 1 week ☐ > 1 week

If greater than 1 week, how long did you experience nausea? _____

Has this symptom resolve itself? ☐ Yes ☐ No

Did you initiate pharmacological treatment for nausea? ☐ Yes:_____ ☐ No

---

☐ Vomiting

☐ 1 - 2 days ☐ 3 - 5 days ☐ 1 week ☐ > 1 week

If greater than 1 week, how long did you experience vomiting? _____

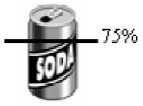
Has this symptom resolve itself? ☐ Yes ☐ No

Did you initiate pharmacological treatment for vomiting? ☐ Yes:_____ ☐ No

---

☐ Constipation

☐ 1 - 2 days ☐ 3 - 5 days ☐ 1 week ☐ > 1 week



If greater than 1 week, how long did you experience constipation? _____

Has this symptom resolve itself? ☐ Yes ☐ No

If No, how many cups of fluid to you ingest per day (1 cup = 75% of 1 can of soda)?

☐ 1 - 2 cups ☐ 3 - 4 cups ☐ 5 - 6 cups ☐ 7 - 8 cups ☐ > 8

Did you initiate pharmacological treatment for constipation? ☐ Yes:_____ ☐ No

---

☐ Increase in evacuation frequency

☐ 1 - 2 days ☐ 3 - 5 days ☐ 1 week ☐ > 1 week

If greater than 1 week, how long did you experience increased evacuation frequency? _____

Has this symptom resolve itself? ☐ Yes ☐ No

Did you initiate pharmacological treatment for increased evacuation frequency?

☐ Yes:_____ ☐ No

---

4. Have experienced any other side effects?

☐ Yes: _____ ☐ 1 - 2 days ☐ 3 - 5 days ☐ 1 week ☐ > 1 week

If greater than 1 week, how long did you experience the above symptom _____

Has this symptom resolve itself? ☐ Yes ☐ No

Did you initiate pharmacological treatment for the above symptom? ☐ Yes:_____ ☐ No

---

PATIENT ENERGY EXPENDITURE CALCULATOR

Patient Total Energy Expenditure Calculator

Mifflin Equation

Patient: Sample **PHASE:** _____

Date (mm/dd/yyyy): ____/____/_____

Men		**RMR = (9.99 x Wt) + (6.25 x Ht) - (4.92 x age) + 5**	
	Wt(kg)	85	
	Ht(cm)	177	
	Age	32	
Resting Metabolic Rate		1802.96	kcal
	Activity Factor	1.7	
Total energy expenditure		**3065**	**Kcal**

Women		**RMR = (9.99 x Wt) + (6.25 x Ht)- (4.92 x age) - 161**	
	Wt(kg)	0	
	Ht(cm)	0	
	Age	0	
Resting Metabolic Rate		-161	
	Activity factor	1.7	
Total energy expenditure		**-273.7**	**Kcal**

Appendix IV

PATIENT DIET CARDS

PATIENT Sample

DAY 1 **PHASE:**

Total Cal 2578.41 kcal /day

BREAKFAST	Grams		Grams
Tropicana Orange Juice BKC	181.5	**Bagel and Jam**	
2% Lowfat Milk-Unf A X Vit D Added TXFI	188.4	Plain Bagel (4 1/2" diameter)	32.7
		Kraft Strawberry Jam KFT	9.4
Cereal			
Kellogg's Corn Flakes RTE Cereal KLC	43.0	**Treatment**	
		Banana Muffin	1 muffin
		Becel - Original	7.3
Scrambled Eggs Dish			
Large Whole FreshEgg-Raw-Each	8.7		
Raw Egg White-Fresh-Cup Measure	98.0		
Tomatoes Chopped/Sliced,Rcd,Raw,Ripe Cu	17.2	**TREATMENT**	
Becel - Original	12.3		
Butter-Salted LOL	3.6		

PATIENT Sample

DAY 1 **PHASE:**

Total Cal 2578.41 kcal /day

LUNCH	Grams		Grams
Apple Juice + Vit C-Cnd/Bottled,Unsw	185.2	**Cucumber and Tomato Salad**	
		Cucumber, Peeled	72.6
Pita Pizza		White Distilled Vinegar	2.9
White Pita Bread-Unenriched-6 1/2"diam	79.9	Olive Oil	2.2
Contadina Pizza Sauce-Original DLM	47.2	Tomatoes Chopped	72.6
Chicken Breast-w/o Skin-Boneless-Roasted	24.0		
Mushrooms-White-Raw MUC	32.7	**Dessert**	
Sweet Green Bell Peppers-Raw-Ring	26.9	RCFFN Carrot Cake	25.4
Back Bacon - Grilled	10.9	Creamy Vanilla Frosting-Cann	8.7
Baby Zucchini Squash-Raw	29.0	Seedless Grapes	18.2
White Onions-Raw-Chopped-Cup	18.2		
Becel - Original	8.7		
Butter-Salted LOL	1.5	**TREATMENT**	
Mozzarella Cheese-Part Skim-Shredded	21.8		

Appendix IV: Sample of patient data cards

PATIENT Sample

DAY 1 **PHASE:**

Total Cal 2578.41 kcal /day

DINNER	Grams
Apple Juice + Vit C-Cnd/Bottled,Unsw	174.3
Chicken Dish	
Chicken Breast-w/o Skin-Boneless-Roasted	58.8
Cranberry Sauce-Canned,Sweetened-Cup	36.3
Butter-Salted LOL	1.5
Peeled Potato-Boiled w/o Skin-Each	72.6
Whole Carrots (7.5" Long)-Raw-Each	32.7
Becel - Orginal	9.4

Treatment	Grams
Apple Muffin	1 muffin
Becel - Original	7.3
Dessert	
Hershey's Chocolate Syrup	18.9
Lo-Fat Frozen Yogurt-Van/Fru	73.8
Saltine Crackers	28.0

TREATMENT _____

ISOTOPE ADMINISTRATION CALCULATOR

Isotope Administration Calculation

DATE (MM/DD/YYYY)	_____/_____/_____	
PHASE:	_____	
Patient	**SAMPLE**	
Weight	91	kg

Deuterium Administration

Body Water (60%)	**54.6**	**kg**
Amt of Deuterium (1.2 g/kg body water)	**65.5**	**g**
	65.5	**ml**

C13 Palmitate Administration

C13 Palmitic Acid Dose 10 mg/kg	**910**	**mg**	
Amount of Margarine	**10.1**	**g**	**90mg Palmitate/g margarine**

Appendix IV

ENERGY EXPENDITURE TRACKING FORM

ENERGY EXPENDITURE TRACKING FORM

Patient: ________		Patient: ________	
Time	Status	Time	Status
___ : ___	ON	___ : ___	On
___ : ___	Breakfast	___ : ___	Breakfast
___ : ___	ON	___ : ___	ON
___ : ___	OFF	___ : ___	OFF
___ : ___	ON	___ : ___	ON
___ : ___	OFF	___ : ___	OFF
___ : ___	ON	___ : ___	ON
___ : ___	OFF	___ : ___	OFF
___ : ___	ON	___ : ___	ON
___ : ___	OFF	___ : ___	OFF
___ : ___	ON	___ : ___	ON
___ : ___	OFF	___ : ___	OFF
___ : ___	ON	___ : ___	ON
___ : ___	OFF	___ : ___	OFF

Appendix IV

COORDINATOR'S NOTES FORM

Coordinator's Notes
PULSE Study 2007

Subject ID:_____ Phase:_____ Date:_____

Subject ID:_____ Phase:_____ Date:_____

Subject ID:_____ Phase:_____ Date:_____

Subject ID:_____ Phase:_____ Date:_____

Subject ID:_____ Phase:_____ Date:_____

CALENDER

July 2007

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
1 Day 28, Phase 1 Group 3 Q: Group 3 wk 4	2 Day 1, Phase 2 Group 1 Day 1, Phase 1 Group 6 Day 29, Phase 1 Group 8	3 Day 2, Phase 2 Group 1 Day 2, Phase 1 Group 5 MW6467 C13 EE 6am	4 Fecal: Group 4 BA5339 C13 EE 6:30 LH6579 C13 EE 7:00	5 AB 3107 C13 EE 6:30 BD5309 C13 EE 7am	6 Fecal: Group 4	7
8 Day 28, Phase 1 Group 4 Q: Group 1, wk1 Q: Group 6, wk1 Q: Group 4, wk 4	9 Day 29, Phase 1 Group 4 Day 1, Phase 1 Group 7	10 Day 2, Phase 1 Group 7 RB4167 EE C13, 6:30	11 Fecal: Group 5	12	13 Fecal: Group 5	14
15 Day 28, Phase 1 Group 5 Q: Group 5, wk 4 Q: Group 7 wk 1	16 Day 29, Phase 1 Group 5 SCREENING	17	18	19	20	21
22	23	2	25 Fecal: Group 1 Fecal: Group 6	26	27 Fecal: Group 1 Fecal: Group 6	28
29 Day 28, Phase 3 Group 1 Day 28, Phase 1 Group 8 Q: Group 1, wk 4 Q: Group 6, wk 4	30 Day 29, Phase 3 Group1 Day 29, Phase 1 Group 6 Day 1, Phase 2 DH1626 Day 1, Phase 2 Group 3	31 Day 2, Phase 3 DH1626 Day 2, Phase 2 Group 3				

Appendix IV

ADVERTISING

Richardson Centre
for Functional Foods
and Nutraceuticals

UNIVERSITY OF MANITOBA

DO YOU WANT TO …

- **LOWER YOUR CHOLESTEROL**
- **IMPROVE YOUR ANTIOXIDANT STATUS**
- **STABILIZE BLOOD SUGAR LEVELS**

If you are:

- 18-60 years old
- Overweight
- No lipid lowering medications

Free Food
&
Financial Renumeration

You might be eligible to participate clinical reasearch trial at

The University of Manitoba's

Richardson Centre For Functional Foods & Nutraceuticals

Unlock The Health Benefits of Food

Contact our Clinical Research Department:

Professor, Peter Jones
(204) 474-9787 or (204) 298-5483
www.RCFFN.ca

Richardson Centre
for Functional Foods
and Nutraceuticals

UNIVERSITY OF MANITOBA

DO YOU WANT TO . . .

- **LOWER YOUR CHOLESTEROL**
- **IMPROVE YOUR ANTIOXIDANT STATUS**
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If you are:

- Male and Female
- 18-60 years old
- Overweight
- No lipid lowering medications

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Unlock The Health Benefits of Food

Contact our Clinical Research Department:

Dr. Peter Jones
(204) 474-9787 www.RCFFN.ca

Richardson Centre
for Functional Foods
and Nutraceuticals

UNIVERSITY
OF MANITOBA

Nutrition Study on Pulses
(Pea, Lentil, Bean, Chickpea)

The Richardson Centre for Functional Foods and Nutraceuticals at The University of Manitoba is conducting a study to investigate the effects of pulses (peas, lentils, beans, chickpeas) on blood cholesterol levels as well as factors related to diabetes, obesity and cancer.

The study is open to healthy men and women who meet the following criteria:

- Aged 18-60 years
- Overweight
- Have elevated blood lipid levels
- Not taking medication to lower triglycerides

Volunteers will be compensated for their participation.

If interested, please call:

(204) 474-9787 or (204) 298-5483
www.RCFFN.ca

Professor Peter Jones, Principal Investigator

Newspaper advertisement

Appendix IV

GENERAL INFORMATION SHEET

General Information sheet

Effect of Pulses and Pulse Fractions on Indices of Lipid, Carbohydrate, and Energy Metabolism, as well as Oxidative Status in Overweight, Hyperlipidemic Individuals

Additional Information Sheet

The Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) is located in the SmartPark at the Fort Garry Campus of University of Manitoba.

General information regarding the study

Pulses are crops yielding 1 to 12 seeds enclosed in pod. They include varieties of beans, lentils, chick peas, and yellow peas. Pulses considered a healthy food choice since they are low in fat and high in fiber, protein, and antioxidants. The purpose of this study is to determine the effect of pulse and pulse fractions on energy expenditure, blood lipids, body composition and antioxidant status. More importantly this study will help determine which fractions of pulses possess the greatest health benefits.

The study will consist of 3 treatment phases, each being one month long. After each treatment phase, there will be a one month break. At this time, you will return to your normal eating habits, and you will not have to come to the centre.

During each phase, you will be asked to come to the centre in the morning between 7 and 9am, (including weekends), to consume breakfast and the specified treatment. The treatment (1/2 cups of pulses/day) will be incorporated into a bread product and consumed with breakfast and dinner. The rest of the day's meals (lunch and dinner) and treatment will be prepared for you at the centre as take out.

The treatments will include; 1. bread products with no pulse flour, 2. bread product containing whole pulse flour and 3. bread products containing only four made from pulse fiber.

Prior to initiating the study, you will also undergo a routine blood, and physical examinations confirm you are of a good health status.

For each phase, on days 1, 2, 28 and 29, you will have blood drawn before breakfast to determine your cholesterol level and other blood values. The total amount of blood drawn during each phase of the study will be approximately 9 tablespoons. On day 28 of each phase, after the blood draw you will be asked to take three tablespoons of tagged water and margarine orally; this is done to allow us to measure how much triglycerides have been produced in the body as well how much as been used a source of energy. On one day during the first and last week of each phase, you will have your resting metabolic rate measured over a 5

hour period. At the same time, we will take breath samples and fingerpick blood samples every 30 minutes. In addition you will have your body fat and non-fat tissue measured by using dual energy x-ray absorptiometry (DEXA). You will be compensated for your participation in the study.

If you are interested in participating in this study, the next step is to come to the RCFFN for an information session where a detailed explanation of the study and the centre will be presented. After attending the information session, you will be invited back to the centre for blood tests to make sure your cholesterol level meets those required for our study. You must have fasted for 12 h and had no alcohol for 24 h before this blood test.

Some possible questions:

Which kind of supper will be served?

A North American diet will be implemented for the duration of the study. Some examples of the types of foods served include; spaghetti, chicken, sandwiches, eggs, stir-fry, and meatloaf. The pulses will be incorporated into bread products as part of the four ingredient. You will be asked to consume breakfast and one treatment per day under supervision at the RCFFN. Lunch, dinner and the remaining daily treatments will be provided as take out.

Is the tagged water dangerous?

No. The tagged water is non-radioactive and non-toxic.

How much is the radiation dose that I will receive for doing the DEXA test?

The amount of radiation that you will receive for the DEXA test per scan is 1% of the radiation dose that you would be exposed to if you were taking an airplane flight across Canada.

If I already know my cholesterol level, can I avoid the 1st blood draw?

No. In order to keep our study controlled, all volunteers have to have their blood tested at the same place, since different laboratories may produce different results.

APPENDIX V

FORMS CORRESPONDING TO CHAPTER IV

PATIENT CONSENT FORM

UNIVERSITY OF MANITOBA

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study: **Palatability and acute glycemic response to pea flour foods**

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

Initials of Subject: _____

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of Study

The purpose of the study is to test the acceptability of baked products made from pea flour with the aim of creating a functional food with optimal glucose regulating properties, which could easily be included in the North American diet.

Study procedures

As an eligible participant, you will have to ensure that you have no history of diabetes, thyroid disease, heart disease, celiac disease or gluten intolerance. You must also make sure that you have not consumed any medication for glycemic control such as insulin or oral glucose lowering agents, or medication and/ or natural health supplements containing substances affecting glucose metabolism such as cortisone, corticotropin and phenytoin for the past three months. In addition, you have to ensure that you do not smoke or consume large amounts of alcohol (>2drinks/day).

If you agree to take part of this study, you will be asked to taste 9 different types of baked food samples (muffins, pasta and biscotti) on your first visit and rate the samples on a hedonic scale, which is a scale measuring the extent of your liking or disliking the food. The procedure will be explained to you by the study coordinator. On your subsequent visits to the center you will be asked to take an oral glucose tolerance test (OGTT) which is used to determine the levels of glucose in your blood before (baseline) and 30, 60, 120 and 240 minutes after the ingestion of each of the test foods. The OGTT is a very simple, fast and minimally invasive procedure which consists in collecting one drop of blood by finger pricking and measuring glucose using an Accu-check Compact plus monitor. The test has to be taken after a 12 hour overnight fast (No food, no drinks other than water). A washout period (break) of two days will be required between each testing day.

The study period will be 26 days, during which you will maintain your habitual diet and exercise routines. However you will be asked to abstain from including pulses (peas, beans and lentils) in your diet and refrain from consuming alcohol. Caffeine-containing beverages will be limited to one cup a day as caffeine was shown to increase glucose levels in the blood.

Risks and Discomforts

There are no risks associated with the study procedures outlined above. However, multiple finger pricks might cause some discomfort in the finger tips. In order to minimize discomfort, we will use different fingers for each test.

Initials of Subject: _____

Benefits
Although you may not benefit from participation in this research, the study will help you make better choices in terms of breads, cereals and baked goods, based on their effect on your blood glucose response. After the study is completed, you will be receiving a report outlining your results in response to each of the test foods consumed.

Costs
All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Payment for participation
You will receive $300 at completion of this study for your time and inconvenience of the study schedule. This amount will be given to you in full on your last visit to the center. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount

Alternatives
You do not have to participate in this study. The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study.

Confidentiality
Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN staff involved with your care may review/copy medical information that may reveal your identity. With your permission, the study doctor will also write to your Family Doctor to tell him/her that you are taking part in a study or to obtain further medical information. The Biomedical Research Ethics Board at the University of Manitoba may also review your research-related records for quality assurance purposes. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the Richardson Centre for Functional Foods and Nutraceuticals.

Voluntary Participation/Withdrawal From the Study
Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff's instructions.

Initials of Subject _____

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially closed.

Medical Care for Injury Related to the Study

In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Kesselman at (204) 954-4486 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Kesselman will be contacted, who will inform you of the results.

Questions

You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

Investigator:	Dr. Peter Jones	Tel No.	204-474-9787
Study Physician	Dr. Edward Kesselman	Tel No.	204-954-4486

For questions about your rights as a research subject, you may contact:
The Biomedical Research Ethics Board, University of Manitoba at 789-3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Consent

I agree to allow the study doctor to inform my family doctor that I am participating in this study or to obtain information regarding my medical history.

Yes ☐ No ☐

1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.

2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.

3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms or changes in my health.

Initials of Subject: _____

4. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.

5. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.

6. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature:_____ Date/Time: _____

Printed name of above:_____

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____ Date/Time: _____

Printed name of above:_____ Study role:_____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

Initials of Subject: _____

Appendix V

PATIENT SCREENING FORM

Pulse food evaluation 2008
Telephone screening form

Date of call:
Name:
Telephone number: (home): (work):
Age:
Birth Date (Y/M/D):
Weight: kg
BMI: kg/m^2
Height: m

Smoker: Y or N
History of heart disease, diabetes, hypertension, hypothyroidism: Y N
Prescription drug intake (daily):
BP medication:
Lipid-lowering medication:
Thyroid medication:
Others:
Have you taken supplements containing plant sterols/phytosterols within last 6 weeks?
Have you taken fish oil capsules in the past 6 months?
Alcohol consumption (glasses/day)
Are you taking multi-vitamin supplements? (multi, Vit E, Vit A, beta-carotene, etc.)
Are you taking herbal or food supplements? (units/day)
Allergies:

Appointment
Blood Test (Fasting)
Medical Exam

Date:
Time:
Date:
Time:
Date:
Time:
Date:
Time:
Eligibility for study:
Start date of study:
Subject Code / Identification Number:

Appendix V

SENSORY EVALUATION FORM

Date: March 3rd, 2008

Subject ID: **SAMPLE**

Product **506**	Dislike very much	Dislike	Neither like nor dislike	Like	Like very much	Comments
Appearance						
Smell						
Taste						
Texture						
Overall						

Product **138**	Dislike very much	Dislike	Neither like nor dislike	Like	Like very much	Comments
Appearance						
Smell						
Taste						
Texture						
Overall						

Appendix V: Sensory evaluation form

Product **613**	Dislike very much	Dislike	Neither like nor dislike	Like	Like very much	Comments
Appearance						
Smell						
Taste						
Texture						
Overall						

Product **345**	Dislike very much	Dislike	Neither like nor dislike	Like	Like very much	Comments
Appearance						
Smell						
Taste						
Texture						
Overall						

Appendix V: Sensory evaluation form

Product **127**	Dislike very much	Dislike	Neither like nor dislike	Like	Like very much	Comments
Appearance						
Smell						
Taste						
Texture						
Overall						

Product **149**	Dislike very much	Dislike	Neither like nor dislike	Like	Like very much	Comments
Appearance						
Smell						
Taste						
Texture						
Overall						

Appendix V

GLYCEMIC RESPONSE FORM

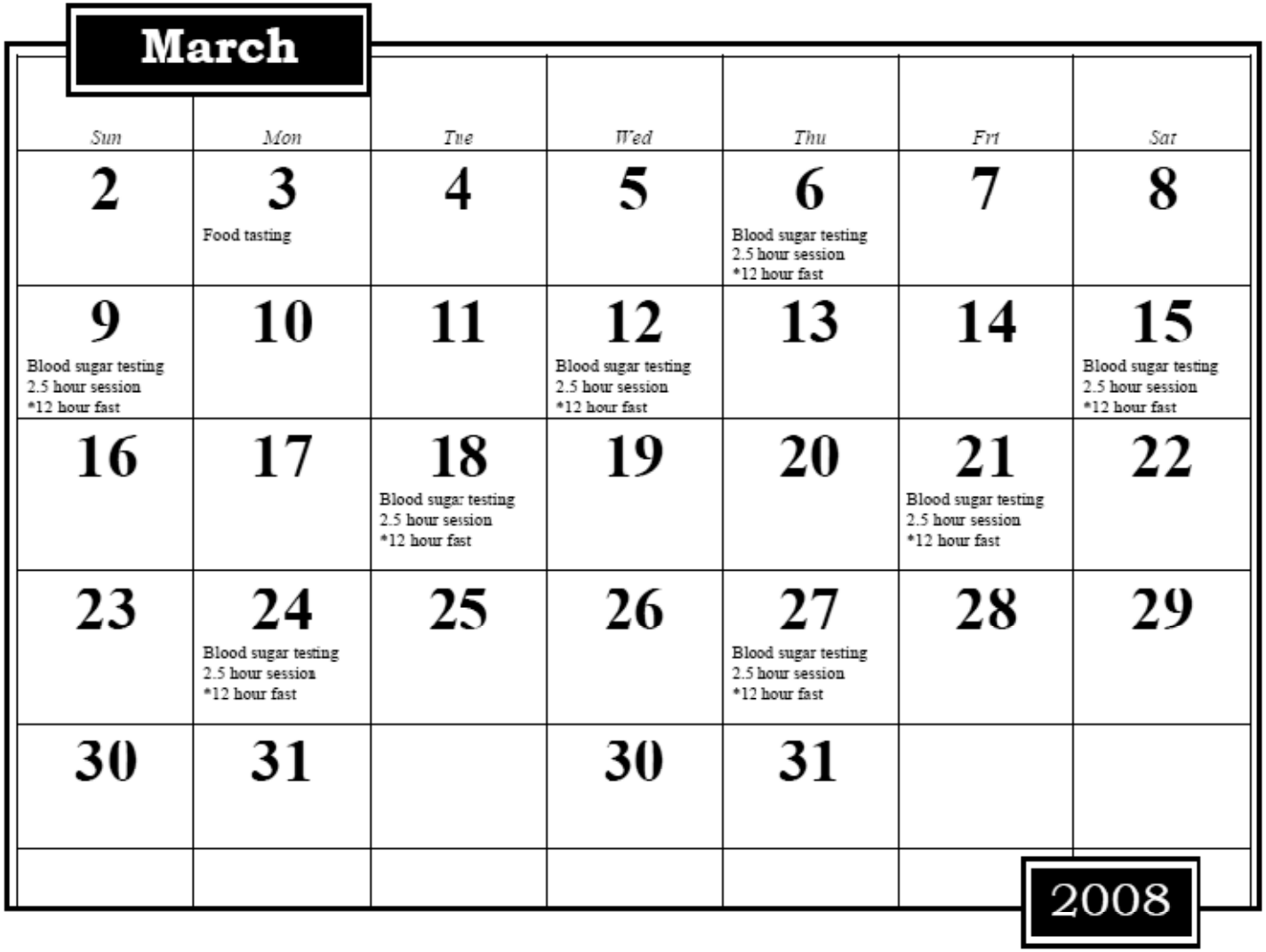
Pulse food evaluation study 2008

Oral glucose tolerance test

Date: Thursday March 6th

Subject ID	Food item ID	Interval/time(min)	Glucose 1	Glucose 2	Glucose 3
SN710	138	0			
		30			
		60			
		120			
		150			
MS711	345	0			
		30			
		60			
		120			
		150			
SP712	137	0			
		30			
		60			
		120			
		150			
RJ713	138	0			
		30			
		60			
		120			
		150			
DG714	138	0			
		30			
		60			
		120			
		150			
JW715	873	0			
		30			
		60			
		120			
		150			

CALENDER



March

Sun	*Mon*	*Tue*	*Wed*	*Thu*	*Fri*	*Sat*
2	**3** Food tasting	**4**	**5**	**6** Blood sugar testing 2.5 hour session *12 hour fast	**7**	**8**
9 Blood sugar testing 2.5 hour session *12 hour fast	**10**	**11**	**12** Blood sugar testing 2.5 hour session *12 hour fast	**13**	**14**	**15** Blood sugar testing 2.5 hour session *12 hour fast
16	**17**	**18** Blood sugar testing 2.5 hour session *12 hour fast	**19**	**20**	**21** Blood sugar testing 2.5 hour session *12 hour fast	**22**
23	**24** Blood sugar testing 2.5 hour session *12 hour fast	**25**	**26**	**27** Blood sugar testing 2.5 hour session *12 hour fast	**28**	**29**
30	**31**		**30**	**31**		

2008

APPENDIX VI

AUTHOR PUBLICATION AGREEMENTS

THESIS PUBLICATION AGREEMENT FOR CHAPTER III

The following acknowledges that I am aware that Christopher Marinangeli will be including the manuscript entitled "Whole and Fractionated Yellow Pea Flours Reduce Fasting Insulin and Insulin Resistance in Hypercholesterolemic and Overweight Humans" in his Doctorate of Philosophy thesis entitled The Effects Of Whole and Fractionated Yellow Pea Flours on Indices of Cardiovascular Disease, Diabetes and Thermogenesis as well as the Gastrointestinal Microbiome.

[redacted]

Dr. Peter J.H. Jones

Sep 14, 2010
Date

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Dr. Amira N. Kassis

[redacted]

Dr. Peter J.H. Jones

Date

Sept 14, 2010

Date

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The following acknowledges that I am aware that Christopher Marinangeli will be including the manuscript entitled "Glycemic Responses and Sensory Characteristics of Whole Yellow Pea Flour Added to Novel Functional Foods" in his Doctorate of Philosophy thesis entitled The Effects Of Whole and Fractionated Yellow Pea Flours on Indices of Cardiovascular Disease, Diabetes and Thermogenesis as well as the Gastrointestinal Microbiome.

[redacted]

Dr. Amira N. Kassis

14/09/2010

Date

Dr. Peter J.H. Jones

Date

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[redacted]

Dr. Peter J.H. Jones

Sept 14, 2010

Date

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Dr. Dennis Krause — Date

[redacted]

Dr. Scott V. Harding — Sept 13, 2010 — Date

[redacted]

Dr. Todd C. Rideout — Sept 13/2010 — Date

Fuqin Zhu — Date

[redacted]

Dr. Peter J.H. Jones — Sep 14, 2010 — Date

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[redacted]

Dr. Denis Krause — SEP 23 - 2010 — Date

Dr. Scott V. Harding — Date

Dr. Todd C. Rideout — Date

[redacted]

Fuqin Zhu — Sept. 23, 2010 — Date

Dr. Peter J.H. Jones — Date

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Dr. Ehsan Khafipour	Date
Fuqin Zhu	Date
Dr. Denis Krause	Date
[redacted]	Sep 29, 2010
Dr. Peter J.H. Jones	Date

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