AMERICAN UNIVERSITY OF BEIRUT

THE EFFECT OF BREAD FORTIFICATION WITH PHOSPHORUS AND LYSINE ON POSTPRANDIAL GLYCAEMIA AND LIPIDEMIA

by
LARA NAIM CHEHADE

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LARA NAIM CHEHADE

Approved by:

Dr. Omar Obeid, Professor — Advisor
Nutrition and Food Sciences

Dr. Imad Toufeili, Professor
Nutrition and Food Sciences — Member of Committee

Dr. Assaad Eid, Associate Professor
Anatomy, Cell Biology and Physiological Sciences — Member of Committee

Date of thesis defense: January 27, 2021

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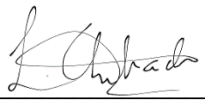

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ABSTRACT
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Many low socioeconomic populations rely mainly on bread as their main source of protein and energy. Wheat contains low quality protein, and thus wheat based diets cannot sustain optimal growth. Moreover, phosphorus found in cereals is mainly in the form of phytate, which is not bioavailable. A previous study done on rats found that supplementing a gluten-based diet with a combination of lysine and phosphorus was able to highly increase weight gain and energy efficiency. Considering that combined effect along with research that relates phosphorus and lysine separately to blood sugar and lipids, the aim of this study was to investigate the effect of white flour fortification with phosphorus and/or lysine on postprandial glycaemia and lipidemia in humans.

Twelve healthy male subjects underwent four different visits. For each visit, participants were randomly given a different kind of bread (fortified with lysine, phosphorus, both lysine and phosphorus, or control). A series of pre- and post-ingestion blood withdrawals were performed, and different blood components including insulin, glucose, triglycerides, and blood urea nitrogen were measured.

Significantly higher levels of insulin and higher levels of Glp-1 that almost reached significance were seen in the control bread when compared with the LP bread paralleled by a reduction in glucose levels. Also, a significant difference in the change of triglycerides was seen between the P bread and the LP bread. These findings imply that the combination of lysine and phosphorus in breads led to an improvement in insulin sensitivity and a trend towards lower triglyceride levels.

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ABBREVIATIONS

~	Approximately
β	Beta
°C	Degree Celsius
>	Greater than
<	Less than
/	Per
%	Percent
µg	Microgram
µU	Microunit
ANOVA	Analysis Of Variance
Apo	Apolipoprotein
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
AUB	American University of Beirut
AUC	Area Under the Curve
BID	bis in die - twice a day
BMI	Body Mass Index
BMR	Basal metabolic rate
BUN	Body Urea Nitrogen
C	Control
dL	Deciliter
DM	Diabetes Mellitus
DIT	Diet-induced thermogenesis
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immunosorbent Assay
et al	And others
FABP	Fatty acid-binding protein
Fig	Figure
g	Gram
GLM	General Linear Model
GLP	Glucagon-Like Peptide
HbA1c	hemoglobin A1c
HDL	High Density Lipoprotein
HIP	Health Insurance Plan
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IVGT	Intravenous Glucose Tolerance
Kcal	Kilocalories
kg	Kilogram
l	Liter
LDL	Low Density Lipoprotein
L	Lysine
LP	Lysine & Phosphorus
MDRD	Modification of Diet in Renal Disease
mg	Milligram
mmol	Millimol
ml	Milliliter

min	Minute
NHANES-WWEIA	National Health and Nutrition Examination Survey—What We Eat in America
ng	Nanogram
OGTT	Oral Glucose Tolerance Test
P	Phosphorus
p	p value
RDA	Recommended Dietary Allowance
RPM	Revolutions per Minute
TDS	Total Diet Study
T	Time
Tr	Treatment
TG	Triglycerides
SCI	Spinal Cord Injury
SE	Standard Error
USDA	United States Department of Agriculture
VLDL	Very Low Density Lipoprotein

CHAPTER I

INTRODUCTION

Diabetes mellitus (DM) is a major public health concern. Worldwide, the prevalence of DM has augmented dramatically. The estimated prevalence of diabetes has risen from 151 million (4.6% of the global population) in the year 2000 to 463 million (9.3%) in 2019. Without sufficient action, it is predicted that this number would increase to 578 million (10.2%) by 2030 (Williams et al., 2019). As a matter of fact, the prevalence of type 2 DM among men in Lebanon was previously recorded to be 26.41% (Meo et al., 2019). If left untreated, diabetes can lead to serious complications such as heart disease, stroke, kidney damage, and nerve damage. Moreover, diabetes has been strongly correlated with hyperlipidemia and other comorbid factors such as hypertension and obesity (Ghassibe-Sabbagh et al., 2014). Metabolic syndrome with its components including impaired glucose tolerance, obesity, and dyslipidemia are also considered a risk factor for type 2 DM. In 2008, the prevalence of metabolic syndrome among Lebanese adults attending health centers was shown to be 31.2% (Sibai et al., 2008). This shows an alarming occurrence and requires well-targeted interventions.

Wheat and wheat derived products are highly consumed around the world. They serve as the main source of protein and energy for many people. Actually, bread is widely consumed in Lebanon, especially white bread. Bread is made from wheat, and wheat is mainly composed of carbohydrates, yet it contains low quality protein and lack some essential amino acids, primarily lysine. Moreover, wheat contains phosphorus in the form of phytate, which greatly decreases the body's ability to absorb it. Therefore, bread would fail to meet human needs and sustain growth.

Fortifying a wheat based meal with a combination of lysine and phosphorus has previously shown positive results in terms of growth and energy efficiency in rats (Ragi et al., 2018). Moreover, a phosphorus pre-load prior to an oral glucose tolerance test revealed a promising trend towards lower insulin response and improved insulin sensitivity in healthy male adults (Abi Rached, 2012). Accordingly, it would be interesting to know if the combination of both lysine and phosphorus would increase the protein quality of the bread, and improve insulin sensitivity by decreasing the insulin and sugar spike after a meal.

Therefore, the principal objective of this study is to investigate if fortifying white bread with lysine and/or phosphorus has a significant effect on postprandial glycaemia and lipidemia in non-obese male subjects.

CHAPTER II

LITERATURE REVIEW

A. Bread

1. Overview and Consumption

Bread is one of the foundations of the human diet as it is highly consumed around the world. It has been around for approximately thirty thousand years (Riskó et al., 2017). It is also widely consumed in Lebanon. The total diet study (TDS) for the Lebanese population in 2010 showed that the Lebanese population consumed an average quantity of 136.8g of pita bread daily (Nasreddine et al., 2010). Another study conducted on a sample of 992 people in Lebanon showed that the most consumed form of bread was white bread (Lebbos et al., 2019). White bread is also the most widely consumed bread in western countries (Pozzo et al., 2015).

2. Composition of Bread

Bread is an essential part of the diet for people globally. Nutritionally, it provides them with energy, carbohydrates, protein, and different micronutrients. A study done in Pakistan showed that wheat flour contributed 58%, 65%, and 59% of the protein intake and 53%, 58%, and 63% of the energy intake of children, women, and men, respectively (Hussain et al., 2004). Wheat, which is a unique cereal suitable for the preparation of bread, is a common gluten-containing grain. Gluten is a generalized term that describes the storage plant proteins found in different cereal grains such as wheat, rye, barley and their derivatives (Riskó et al., 2017). Plant proteins are incomplete proteins. Thus, they do not contain all the essential amino acids needed to

maintain optimal body functions. In gluten, lysine is considered the limiting essential amino acid (Brody, 1999; Friedman, 2004). Being the essential amino acid means that the rate of protein synthesis depends on the availability of lysine. Additionally, phosphorus found in grains is found in the form of phytate, which makes it largely unavailable. This is due to the insufficient capabilities in the gastrointestinal tract of mono-gastric organisms to degrade it (Bohn et al., 2008). Moreover, research has shown that phytate significantly inhibits the absorption of iron, zinc calcium, magnesium and manganese (Gupta et al., 2015). Therefore, fortifying bread with lysine and phosphorus may increase their bioavailability.

Grain refinement refers to the removal of one or more of the three key parts of the grain, which are the bran, germ, and endosperm. White flour falls under the category of refined grains. It was noted that if the flour is refined, it contains more starch and less nutrients (proteins, lipids, and minerals), dietary fiber, B vitamins and other antioxidants (Nogala-Kałucka et al., 2020). Slavin et al. demonstrated that there is a clear compositional difference in phytate phosphorus content of whole wheat and refined wheat, 2.9 mg/g and 0.1mg/g respectively (Slavin et al., 1999). Moreover, Heshe et al. proved that the protein, fat, fiber, ash, iron, zinc, phosphorous, and antioxidant contents of the refined samples significantly decreased by milling (Heshe et al., 2015). Even though the bioavailability of phosphorus from whole grains is low, interestingly, the American Kidney Foundation, the American Kidney Fund, the Academy of Nutrition and Dietetics, and the Mayo Clinic all recommend avoiding whole grains due to its phosphate content (Williams et al., 2013).

B. Lysine

1. Overview

Lysine is an essential amino acid that has a lot of functions. It is considered a building block as it works with other nutrients to build proteins. It is needed for normal growth and helps with muscles turnover. It also helps the body better absorb calcium for bone health, and is used to make the crucial compound carnitine.

L- Carnitine is an organic nutrient that is required for the entry of long chain fatty acids into the mitochondria. An important function of lysine is to act as a precursor of carnitine as it provides the carbon backbone of carnitine synthesis (Harpaz, 2005; Li et al., 2014). A decreased carnitine synthesis might result in increased lipid accumulation due to reduced mitochondrial fatty acid oxidation.

High-carnitine-fed fish showed an increase in eicosapentanoic acid, a primary liver fatty acid, by 7 % when compared with low-carnitine fish (Ozório et al., 2001). A middle chain fatty acids + L-carnitine dietary intervention lead to a 40% reduction in intramyocellular lipids (Vasiljevski et al., 2020). Nevertheless, it is still controversial whether the supplementation of lysine would affect carnitine levels. Oral administration of 5 g of lysine to six healthy male volunteers increased plasma trimethyllysine (a lysine derivative and carnitine precursor) and plasma and urinary carnitine when compared to administration of tryptophan (Vijayasarathy et al., 1987). Carnitine biosynthesis was further investigated by supplementing adults with excess amounts of carnitine precursors including lysine and methionine, trimethyllysine, or butyrobetaine. A small increase in carnitine production was seen with the lysine and methionine treatment (Rebouche et al., 1989). In Atlantic salmon, a 3 months low lysine diet reduced hepatic carnitine without affecting muscular carnitine when compared to an adequate lysine

diet. Moreover, the fatty acid profiles and lipid classes were unaffected between the different treatments (Rathore et al., 2010). Another study showed that rats fed high lysine diets had significantly higher concentrations of free trimethyllysine in skeletal muscle and plasma relative to control rats and rats fed the high potassium diet (Davis et al., 1993). Also, a study showed that lysine restrictions limited the synthesis of trimethyllysine in rats, yet did not impair the biosynthesis of carnitine (Davis, 1990).

2. Lysine and Growth

Lysine is important for normal growth and muscle turnover. Research has previously shown the effect that lysine fortification has on growth parameters. During a 3 month period, significant greater gains in weight and height were noted in children who consumed lysine fortified flour when compared to those who consumed unfortified flour (Hussain et al., 2004). In addition, fish fed a low-lysine diet had reduced growth, protein deposition, and energy wasting as compared with fish fed the adequate lysine diet (Rathore et al., 2010). Moreover, when comparing different lysine containing diets, maximum growth performances were observed in fish fed with lysine at 19 g/kg of diet when compared with fish fed with lysine at 14.3, 16, 17.5, and 20.5 g/kg of diet (Prabu et al., 2019). When comparing the effect of free amino acid lysine and some more amino acids on weight in streptozotocin-induced diabetic rats, maximum weight increase was seen in the lysine-fed group when compared to the diabetic control group (Sulochana et al., 1998).

3. Lysine and Glycemia

Glycemia refers to glucose that is present in the blood. Abnormal levels of blood glucose can cause severe complications in the body. Hyperglycemia, or high blood sugar levels, can damage blood vessels. In fact, hyperglycemia is a defining characteristic of diabetes. It can also can increase the risk of heart disease, stroke, kidney disease, vision problems, and nerve problems (Giugliano et al., 2008). Jordi et al., has suggested l-lysine for use as one of the novel antidiabetic nutrients as it is a cheap natural product, can be ingested orally, act immediately, and does not require dietary adaptation (Jordi et al., 2014). Moreover, lysine has been shown to have a potent anorectic and a longer satiety effect. A study showed that in humans, L-lysine dose dependently delayed gastric emptying by a rate of 4 min/g L-lysine (Baruffol et al., 2014). By delaying gastric emptying, L-lysine can possibly help moderate the gradual release of glucose into the blood. An oral glucose tolerance test (OGTT) on four-hour food-deprived rats with 6.7mmol/kg of L-lysine showed a reduction in postprandial hyperglycemia already at 15 min post-application when compared to other amino acids (Jordi et al., 2014). Another study reported that intragastric infusion of lysine 15 minutes prior to ingestion of a mixed nutrient drink significantly decreased blood glucose and plasma insulin at 60 min compared with the control (Ullrich et al., 2017). Moreover, a daily 2 ml of 2% solution of lysine supplement given orally to diabetic rats significantly lowered blood sugar levels by 64.76% when compared to the 29.35% decrease seen in the control group (Sulochana et al., 1998). This was further supported when a 2 month lysine oral supplementation decreased fasting blood sugar levels by 27% in type 2 diabetic patients when compared to the non-diabetic healthy control subjects (Sulochana et al., 2001).

4. Lysine and Insulinemia

Insulin is a hormone that is produced by the pancreas as result of high blood sugar levels. It is required to allow glucose to enter the cells in the body. Sener et al., suggested that lysine might affect insulin secretion and cells metabolism after lysine was rapidly taken up by pancreatic islets β cells removed from fed albino rats which resulted in higher concentration of lysine intracellularly than extracellularly (Sener et al., 1989). Another study showed that when 1mol lysine/kg was given with 25g glucose to 13 healthy adults, it attenuated the glucose area response by 44% without an accompanying increase in insulin concentration when compared with the 25g glucose ingestion alone (Kalogeropoulou et al., 2009). Nevertheless, a study showed that the ingestion of 1200mg of lysine with 1200mg of arginine resulted in a 65% increase in insulin from baseline at 30 minutes (Isidori et al., 1981). Intragastric infusion of lysine 15 minutes prior to ingestion of a mixed nutrient drink significantly decreased plasma insulin at 60 min compared with the control (Ullrich et al., 2017). In addition, a study done on spinal cord injured rats demonstrated that a high dose (621.5 mg/kg) of L-Lysine monohydrochloride significantly increased serum insulin and significantly decreased blood glucose when compared with the model control group (Zhang et al., 2010). In contrast, a study that evaluated the impact of acute lysine supplementation found no difference between the control treatment, the glucose and 2g lysine treatment, and glucose and 5g lysine treatment on insulin sensitivity (Kim et al., 2014).

C. Phosphorus

1. Overview

Phosphorus is an essential micronutrient. It being an essential micronutrient means that the body cannot manufacture it in sufficient amounts on its own. Therefore, phosphorus needs to be supplied by the diet. The Recommended Dietary Allowance (RDA) of phosphorus is 700 mg/d for adults of 19 years of age and older (Calvo & Uribarri, 2013a). The analysis of the National Health and Nutrition Examination Survey—What We Eat in America (NHANES WWEIA) cycles from 2001 to 2014 estimated the mean dietary phosphorus consumption for the period between 2001 and 2014 to be 1373 mg/day (McClure et al., 2017). Interestingly, a review comparing phosphorus levels and obesity mentioned that the RDA of phosphorus is based on the lower end of the normal adult serum inorganic phosphate and that it would have been 2,100 mg/day had it been based on the middle of the normal range (Obeid, 2013).

Organic phosphorus is found naturally in protein-rich foods such as meats, poultry, fish, nuts, beans and dairy products (USDA). It can also be found inorganically from food additives and in dietary supplements (Calvo & Uribarri, 2013a; Calvo & Uribarri, 2013b). According to NHANES WWEIA of 2001 to 2014, grains were the largest dietary phosphorus source, followed by meats, and milk (McClure et al., 2017). This raises a question since phosphorus found in animal foods is absorbed more easily than phosphorus found in plant foods. Phosphorus from meat and dairy sources is estimated to be absorbed at approximately 60%, whereas plant-based phosphorus at less than 40% (Calvo & Uribarri, 2013b). Besides, phosphorus that is found in grains is found in the form of phytate. In plants, phytate is the major storage form of phosphorus (Skoglund et al., 2009). In the human body, phytate is not bioavailable as there is a lack

of the enzyme phytase which helps metabolize phytic acid and release the bound phosphorus (Wodzinski and Ullah, 1996). As a matter of fact, the relationship between bread leavening and phosphorus bioavailability has been previously discussed. A study that compared the effects of different kinds of bread fermentation on mineral bioavailability showed that the unfavorable effects of phytic acid in yeast and sourdough bread were lower than in whole wheat flour, ~52% and ~71%, respectively (Lopez et al., 2003). Another study showed that unleavened whole meal bread contained little acid-soluble phosphorus when compared with the production of acid-soluble phosphorus in whole meals of 75% - 85% and 85% - 90% extraction where phytate was being destroyed rapidly by yeast fermentation (Reinhold, 1975). Adults have ~700 g of total body phosphorus stores. Of those stores, 85% reside in bone as hydroxyapatite, 14% are found intracellularly, and the remaining 1% is in extracellular fluid which includes circulating inorganic phosphate (Moorthi & Moe, 2011).

2. Phosphorus, Growth, and Energy

Adenosine triphosphate (ATP) is an organic compound that carries energy in cells of all living things. It is required for many processes such as growth, body temperature maintenance, muscle contractions, and nerve impulses. The energy for these processes is obtained when a phosphate group is removed from ATP to form adenosine diphosphate (ADP) (Lerner & Lerner, 2008). Therefore, sufficient amounts of phosphorus are needed to assure the production of ATP. An animal study showed that energy efficiency among the rat group fed a gluten diet with added phosphorus was significantly higher than that of the control group (Ragi et al., 2018). Moreover, diet-induced thermogenesis (DIT), which is the increase in energy expenditure above the

basal resting rate that occurs after eating, is believed to be largely related to ATP production. A 500mg supplementation induced a 23% increase in DIT area under the curve of overweight/obese subjects, which was associated with a significant increase in carbohydrate oxidation (Bassil & Obeid, 2016). Another study showed that the postprandial energy expenditure of healthy lean males given high protein meals with 500mg of phosphorus was found to be higher than that of the control group (Abdouni et al., 2016).

According to Holliday, the three different components of energy requirement are basal metabolic rate (BMR), energy spent for physical activity and in cold temperatures, and energy used for growth. As a matter of fact, as much as 40% of total energy requirement is used for growth during the early months of life (Holliday, 1986). Since phosphorus is necessary for energy availability, it is believed that it has a great influence on growth as well. A study compared the effect of different diets formulated to contain various phosphorus levels in fish. Results showed that the specific growth rate and weight gain were all significantly improved by dietary phosphorus up to 9.4 g/kg and then levelled off beyond this level when compared to other diets (Shen et al., 2017). Another study reported that phosphorus supplementation of a low-protein diet reduced plasma urea nitrogen and increased total body protein content. Body weight also increased with the increased dietary phosphorus content (Hammoud et al., 2017). Moreover, a rat study reported a significant 5–time increase in total weight gain with the presence of phosphorus in the diet when compared to the control diet (Ragi et al., 2018).

3. Phosphorus and Insulinemia

High carbohydrate ingestion is known to initiate insulin release to ease the uptake of sugar into the cell. It has been proven in research that insulin has a significant relationship with phosphorus. Phosphorus plays a great role with insulin release, sensitivity, and glucose uptake into the cell. Phosphorus treatment for 12 weeks on 63 healthy adults showed a mild but significant difference in changes of insulin and HOMA-IR between the phosphorus and the placebo group (Ayoub et al., 2015). A higher clearance of glucose was previously correlated with a higher clearance of inorganic phosphorus. A significant negative correlation was observed between serum phosphate levels and insulin and HOMA-IR (Park et al., 2008).A study on cows looked at the relationship between insulin and inorganic phosphorus during intravenous glucose tolerance (IVGT) test. The test showed that the more intensive the insulin response during the IVGT test, the greater the decrease in organic phosphorus concentration (Cincović et al., 2017). Simultaneously, insulin exerts an effect on phosphorus. An increase in the level of insulin in the blood decreases serum inorganic phosphorus concentration during IVGT test (Cincović et al., 2017). A study showed that increased insulin levels promote the transport of both glucose and phosphate into skeletal muscle and liver (Liamis et al., 2010). Furthermore, Insulin infusion can cause an abrupt fall in serum phosphate due to intracellular shifts of phosphorus (Pappoe & Singh, 2010). Interestingly, a study showed that low phosphorus diet (0.2% of P) significantly enhanced insulin sensitivity in both male and female mice when compared to the control diet (0.4% of P) (Lin et al., 2018).

Nevertheless, insulin resistance plays a role in postprandial ATP production (Bassil & Obeid, 2016). A study showed that rates of mitochondrial phosphorylation in

skeletal muscles were significantly lower by approximately 30% insulin-resistant subjects when compared to the control subjects (Peterson et al., 2004). Another study showed that insulin-resistant overweight and normal-weight children had significantly prolonged recovery of phosphocreatine, a substance that helps recycle ATP, when compared with the insulin-sensitive children (Fleischman et al., 2009).

4. Phosphorus and Glycemia

Most of the body cells use glucose for energy synthesis. It is the only fuel normally used by brain cells. Research repeatedly demonstrated a correlation between phosphorus and postprandial glucose. Serum phosphorus decreased following glucose ingestion. Venkataraman et al. reported that serum phosphorus values declined significantly after a 1.77 g/kg of glucose ingestion in 10 healthy term infants (Venkataraman et al., 1986). An oral glucose load promoted a significant gradual decrease in serum phosphorus levels with maximum decrease at 120 minute in post-menopausal women (Polymeris et al., 2011). A study showed that co-ingestion of phosphorus with glucose significantly improved postprandial glucose, insulin, and insulin sensitivity index, while phosphorus pre-ingestion failed to exert similar effect. Moreover, ingestion of P alone increased serum P significantly, while ingestion of glucose alone decreased postprandial serum P levels (Khattab et al., 2015)

On the other hand, a study determined that an OGTT of 75g glucose had no significant effect on serum phosphorus. Moreover, there was no significant difference in the mean serum phosphorus levels between subjects with and without impaired glucose tolerance (Baser et al., 2013). In addition, a double-blind, randomized, placebo-controlled trial of 63 adults showed that phosphorus supplementation for a period of 12

weeks did not affect serum glucose (Ayoub et al., 2015). Also, another study where rats were fed protein diets that were fortified with lysine, phosphorus, and lysine and phosphorus showed no difference in plasma glucose concentrations when compared to the control group (Ragi et al., 2018).

5. Phosphorus and Lipids

Lipids are essential to the body. They are needed for energy storage, temperature maintenance, cholesterol formation, and protection against injuries, as well as other functions. Dyslipidemia occurs when one has abnormal levels of lipids in the blood. Dyslipidemia is an important risk factor for coronary artery disease and stroke (Kopin & Lowenstein, 2017). There has been a lot of interest regarding the relationship between phosphorus and blood lipids in research. A study showed that the ingestion of 500 mg of phosphorus with a high fat meal was able to significantly increase postprandial concentrations of apolipoprotein B-48 and decrease those of apolipoprotein B-100, yet demonstrated no significant change of non-esterified fatty acids and triglycerides when compared to the ingestion of placebo (Hazim et al., 2014). In addition, levels of low-density lipoprotein cholesterol, High-density lipoprotein cholesterol, and triglycerides were not affected when placebo and phosphorus supplements were given for 12 weeks (Ayoub et al., 2015). Another study showed that preloading 500 mg phosphorus 60 minutes before a 75 g glucose solution yielded no significant difference between serum triglyceride levels when compared to the placebo (Abi Rached, 2012). Ragi et al. reported that rats fed phosphorus-fortified protein diets maintained significantly lower low-density lipoprotein cholesterol concentrations when compared to lysine-fortified and control diets. Interestingly, the highest levels of serum

triglycerides were detected in rats fed diets fortified with both lysine and phosphorus when compared to the rats of other groups (Ragi et al., 2018). Epididymal fat pad mass was found to be significantly greater in Apolipoprotein E knockout mice that were fed a low 0.2% phosphate diet when compared to the higher 1.6% phosphate diet group (Ellam et al., 2011). A study looked at the effects of six purified diets containing graded levels of phosphorus in young taimen fish. Results showed that whole body lipid content linearly decreased with increasing dietary P levels. Moreover, TG contents of the phosphorus groups significantly decreased when compared with the control group (Wang, et al., 2017). Another study done on puffer fish indicated that dietary phosphorus supplementation could improve lipid transportation since 0.8% phosphorus supplementation significantly upregulated two lipid transport proteins, apolipoprotein A-I and fatty acid-binding protein (FABP) (Ye et al., 2015).

6. Phosphorus and diseases

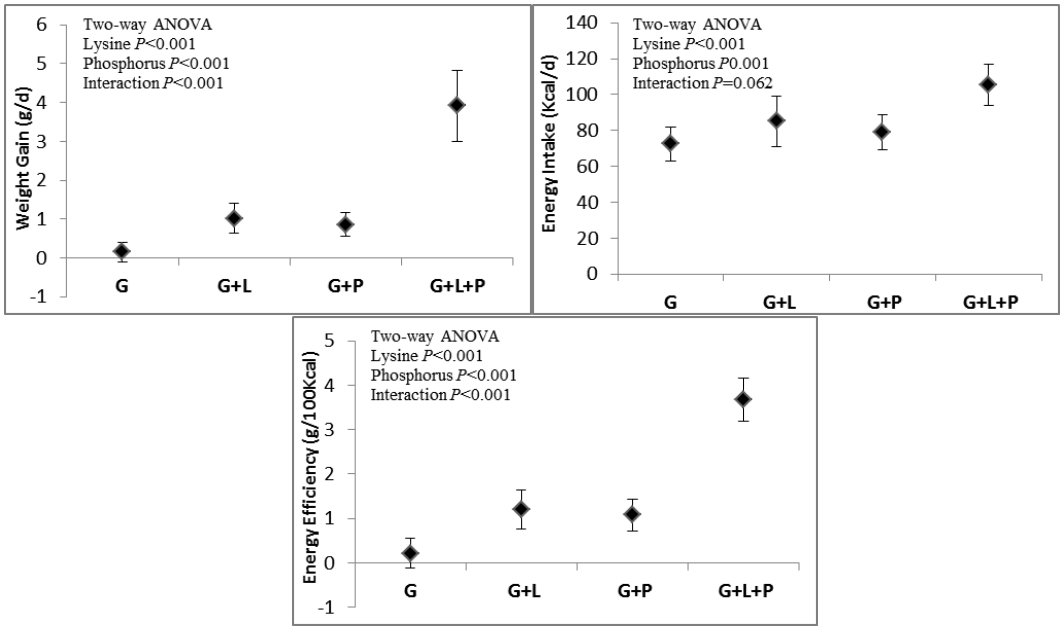
Abnormal levels of phosphorus have been associated with increasing the risk of various diseases, mainly cardio vascular disease, over time. An increase of 0.1 mg/dL of serum phosphate almost doubled the risk of arterial stiffness in newly diagnosed hypertensive patients (Sciacque et al., 2015). A study that evaluated 3368 Framingham Offspring study participants reported that values of serum phosphorus higher than 3.5 mg/dL were associated with a 55% increased cardio vascular disease (Dhingra et al., 2007). Another study done in Korea on 46,798 subjects showed that a high phosphate level is correlated with cardiovascular disease while a lower phosphate level is correlated with metabolic syndrome (Park et al., 2008). Moreover, serum phosphate levels between 1.25 and 1.50 mmol/l were associated with increased cardiovascular

events in people with normal renal function and with stages 1 and 2 Chronic Kidney Disease while hypophosphatemia was associated with fewer cardiovascular events in people with normal renal function (McGovern et al., 2013). In discussions of whether phosphorus supplementation plays a role in preventing such diseases, research has shown controversial results. Apolipoprotein E knockout mice that were fed an atherogenic diet with a high 1.6% phosphate content experienced a significant 40% increase in atheroma by the end of the trial when compared to the mice that were fed a low 0.2% phosphate containing diet. Concurrently, insulin resistance was increased 4-fold and hepatic steatosis was induced in the low phosphate diet when compared with the normal and high phosphate diets (Ellam et al., 2011). Shuto et al., demonstrated that a meal containing 1200mg of phosphorus increased serum phosphorus at 2 h post-ingestion and significantly decreased flow-mediated dilation of the brachial artery in 11 healthy men (Shuto et al., 2009). Disordered eating patients treated with 500mg BID phosphorus had elevations in liver enzymes at presentation or during refeeding and a longer length of stay prior to medical stabilization when compared to patients treated with the 250mg BID dose (Sieke et al., 2017). A study done on 60 healthy subjects showed that supplementation of tri-calcium phosphate and cholecalciferol increased fecal calcium and phosphorus excretion and had no beneficial effect on bone remodeling markers (Trautvetter et al., 2014).

A 2017 study performed on forty male Sprague-Dawley showed that the addition of lysine or phosphorus to wheat gluten-based diets increased mean total weight gain by approximately 5 times, while the addition of lysine and phosphorus together amplified this effect to 20 folds when compared with the control group (Fig. 1). Similarly, the magnitude of improvement in energy efficiency by the addition of lysine

or phosphorus was much lower than that of the combination (Fig. 1) (Ragi et al., 2018). In other words, the addition of phosphorus and lysine separately to an exclusive wheat gluten diet exhibited similar increases in weight gain and food intake. However, the presence of lysine and phosphorus together had a greater impact and was able to further improve growth.

Figure 1 Average daily energy intake, weight gain and energy efficiency of the four groups of rats over the 9-week experimental period



*Two-way ANOVA performed, with lysine and phosphorus as the two variables, and the interaction lysine x phosphorus. Significance is found with P-value < 0.05. Retrieved from Ragi et al., 2018

Considering the multiplied effect shown in mixing phosphorus and lysine on weight gain and energy efficiency, and previous research relating phosphorus and lysine separately to glycaemia, insulinemia, and lipidemia, we are looking to further assess the effect of their combination in bread on postprandial glycaemia and lipidemia, and other blood and urine parameters in 12 healthy human male subjects of normal and overweight BMI.

CHAPTER II

MATERIAL AND METHODS

A. Subjects:

Twelve non-obese (body mass index <30) healthy male participants whose ages ranged from 20-40 participated in the study. The participants were recruited from the American University of Beirut located in the city of Beirut, Lebanon. Individuals with major medical disorders were excluded, as well as those with no health insurance plan (HIP). After a detailed explanation of the study, all participants signed a written informed consent. This study was approved by the Institutional Review Board Committee of the American University of Beirut (BIO-2017-0278).

B. Pre-study visit:

Individuals interested in participating were asked to come in for a pre-study visit. After a thorough explanation of the study, individuals were asked to sign the consent form (Appendix I). Afterwards, anthropometric measurements such as weight and height were measured to calculate BMI. A general health questionnaire (Appendix II) was also filled out to make sure that none of the potential participants had any diseases. One blood sample was collected to determine creatinine values (mg/l), in order to calculate the Estimated Glomerular Filtration Rate (eGFR) using the abbreviated Modification of Diet in Renal Disease (MDRD) equation in order to make sure that the potential participants had normal kidney function (National Institute of Health, n.d.). Individuals with a serum creatinine values >1.2mg/dL and eGFR <60 ml/min/1.73 m^2 were excluded from the study. In addition, a finger prick was done to test for

hemoglobin A1c (HbA1c) to make sure that the potential participants had normal glucose levels. Individuals with HbA1c levels of 6.5% or more were excluded. Testing for HbA1c was done using the Siemens DCA 2000 Analyzer (Erlangen, Germany). Once participants fit the criteria, they were asked to maintain their regular dietary and physical activity habits during the entire study course, and avoid alcohol consumption and any unusual strenuous exercise in the 24 hours prior to their visit.

C. Bread Preparation

All bread used for the experiment was prepared at the Pilot Plant located at the American University of Beirut. To every 500g of white flour; 280ml of water, 5g of yeast, 8g of salt, and 15g of sugar were added. Regarding fortification rates, 625mg of L-lysine monohydrochloride was added to 100g of unfortified white wheat flour, giving a fortification rate of 500mg of lysine in 100g of flour. Also, 500 mg Potassium Phosphate was added to 100g of unfortified white wheat flour. Taking into consideration the 108mg of phosphorus initially present in the 100g of the unfortified white wheat flour, this will add up to a total of 608mg of phosphorus/100g of fortified white wheat flour. Ingredients were mixed in a dough mixer. After that, dough underwent fermentation for 15 mins, rounding and shaping, proofing for 15 minutes, and finally baking at around 500°C using the Arabic Bread Oven. Using a bomb calorimeter, it was determined that 100 grams of bread yields approximately 300 kcals.

D. Experimental Design:

On the experiment day, participants were asked to come in after a 10-hour fast. They were asked to come in approximately at the same time for each visit. After

emptying their bladder, participants were measured for height and weight and a baseline blood withdrawal was performed using a 20-gauge needle inserted into the antecubital vein. All body measurements were taken using the same equipment. Body weight was measured using the Seca 770 electronic scale and height was measured using the Seca 213 stadiometer. The participants were then given 15 minutes to consume 100 grams of bread and water ad libitum. The experiment had 4 different types of bread and they are the following:

- Unfortified control bread (C)
- Bread that was fortified with 0.5% Lysine in the form of L-lysine Monohydrochloride (L)
- Bread that was fortified with 0.5% phosphorus in the form of Potassium Phosphate (P)
- Bread that was fortified with 0.5% Lysine and 0.5% phosphorus (L+P).

Blood was then withdrawn at minutes 15, 30, 45, 60, 90, and 120 post-ingestion and a urine sample was collected at the end of each visit. Each participant came in for four different visits with the bread type being the difference between each visit. The types of breads were administered to the participants in random order with a minimum of 5 days in between as a washout period. The blood samples collected were centrifuged at 3500 RPM for 10 minutes at 4°C and plasma aliquots were gathered and stored at -80°C to be stored for later analysis.

E. Serum and Urine Analysis:

Serum was tested for levels of glucose, insulin, Apolipoprotein B100, Apolipoprotein B48, triglycerides, cholesterol, total phosphorus, high-density

lipoprotein (HDL), blood urea nitrogen (BUN), and Glucagon-Like Peptide-1 (GLP-1). Urine was tested for urea nitrogen, creatinine, and phosphorus.

1. Insulin:

Serum insulin was analyzed using the Millipore Human Insulin ELISA Kit (EZHI-14K) (EMD Millipore Corporation, Bedford MA, USA).

2. Glucagon-Like Peptide-1:

Serum GLP-1 was analyzed using the Millipore Human GLP-1 Total ELISA Kit (EZGLP1T-36K) (EMD Millipore Corporation, Bedford MA, USA).

3. ApoB100 and ApoB4:

Serum Apo B-48 and Apo B-100 were analyzed using My BioSource Human Apolipoprotein B-48 ELISA Kit (MBS166742) and My BioSource Human Apolipoprotein B-100 ELISA Kit (MBS770473) (My BioSource, San Diego CA, USA).

4. Serum Glucose, triglycerides, Total Phosphorus, BUN, Cholesterol, HDL:

Glucose, triglycerides, Total Phosphorus, BUN, Cholesterol, and HDL were determined using the enzymatic colorimetric method on Vitros 350 Chemistry System (Ortho-Clinical Diagnostics, Johnson & Johnson, New York).

5. Urine urea nitrogen, creatinine, and phosphorus:

Urea nitrogen, creatinine, and phosphorus were determined using the enzymatic colorimetric method on Vitros 350 Chemistry System (Ortho-Clinical Diagnostics, Johnson & Johnson, New York).

F. Statistical Analysis

Statistical analysis was performed using IBM SPSS statistics 20 software program. Results were analyzed using the General Linear Model, with treatment and time as variables, and the interaction representing treatment x time, and One-way ANOVA. The probability of less than 0.05 was considered significant.

CHAPTER IV

RESULTS

A. Subject Characteristics

A total of 17 participants were recruited for the study. Three subjects withdrew due to personal reasons and two subjects were excluded, one due to a high BMI level and one due to suffering from hypertension. Twelve participants continued with the study.

Subjects' baseline characteristics including the subjects' age, weight, height, and BMI are summarized in Table 1. Subjects had a mean BMI characterized as overweight (26.2 ± 2.9). The 4 different breads were tolerated by all subjects. However, more than one participant expressed a little bitter taste with the phosphorus bread and that the breads in general needed a little more salt. Moreover, more than one participant mentioned that the breads were a little chewy for their liking.

Table 1 Subject characteristics; mean ± SE

	Subjects (n=12)
Age (years)	25.17 ± 1.75
Weight (kg)	83.62 ± 3.54
Height (m)	1.78 ± 0.02
BMI (kg/m^2)	26.23 ± 0.85

B. Serum Components

1. Insulin

The absolute insulin levels are shown in Figure 2. There was a significant difference between the control bread and the LP bread ($p=0.026$). Also, there was a significant time effect ($p<0.001$). However, there was no significant difference in the

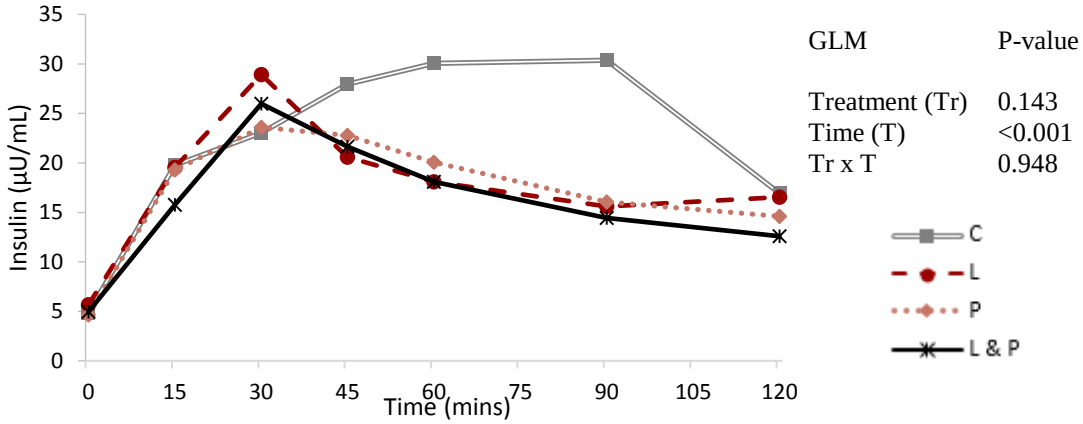
interaction between treatment and time ($p = 0.948$). At minute 90, insulin levels were significantly higher with the control bread (30.37 ± 6.48 μU/mL) than with the LP bread (14.43 ± 2.42 μU/mL) ($p < 0.05$). After minute 30, all three of the treatment breads started decreasing except, the control bread kept increasing until it reached a peak at minute 90 (30.37 ± 6.48 μU/mL).

The postprandial changes in insulin levels from 0 were also assessed (Figure 3). When comparing treatments, a difference that almost reached statistical significance was seen ($p = 0.072$). There was also a significant time effect ($p<0.001$). However, there was no significant significance when looking at the treatment x time interaction ($p = 0.874$).

There was a significant difference between the control bread and the LP bread ($p = 0.01$). Significant difference between the insulin levels in the control bread (25.50 ± 6.28 μU/mL) and the LP bread (9.47 ± 2.13 μU/mL) was seen at minute 90 ($p < 0.05$). The mean area under the curve for insulin was the highest following the control bread consumption (2355 ± 512) when compared to the rest of the breads, though significance was not reached ($p=0.276$) (Table 2, Figure 4).

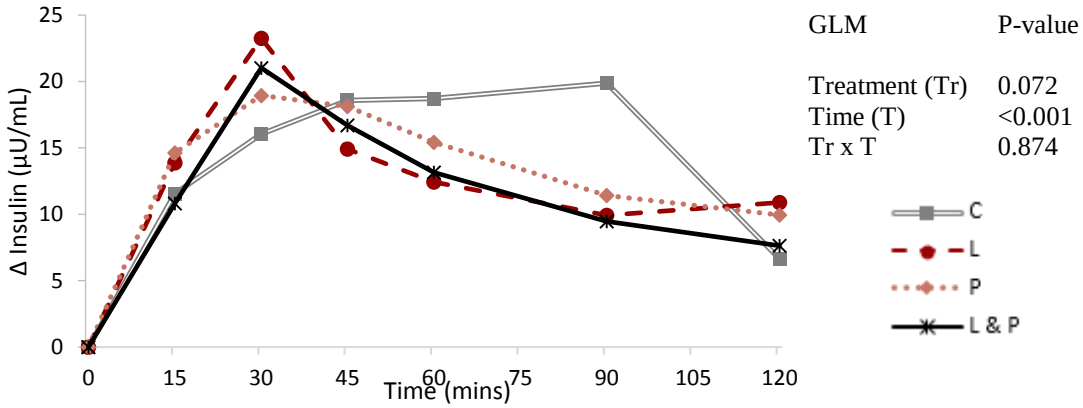
The highest peak was seen at minute 30 with the lysine treatment (28.92 ± 9.83 μU/mL) when compared with the lysine x phosphorus treatment (21.02 ± 4.39 μU/mL) but this difference was not significant ($p=0.245$).

Figure 2 Absolute insulin levels for the different treatments over 120 minutes



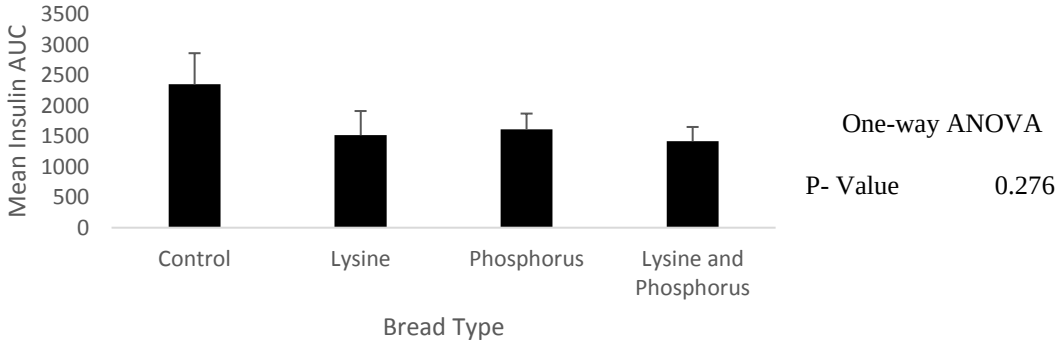
Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 3 Changes in insulin levels from zero for the different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 4 Mean area under the curve of insulin for different treatments



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

Table 2 Mean area under the curve values of certain blood components after different treatments

	Control	Lysine	Phosphorus	Lysine and Phosphorus	P-value
Insulin	2355 ± 512	1521 ± 396	1614 ± 261	1422 ± 233	0.276
Glucose	1438 ± 507	1270 ± 326	978 ± 294	901 ± 341	0.717
GLP-1	-324 ± 159	-333 ± 68	-342 ± 166	-213 ± 247	0.946
Triglyceride	1399 ± 972	846 ± 1215	1933 ± 1166	33 ± 990	0.648

Values are listed as mean ± SE

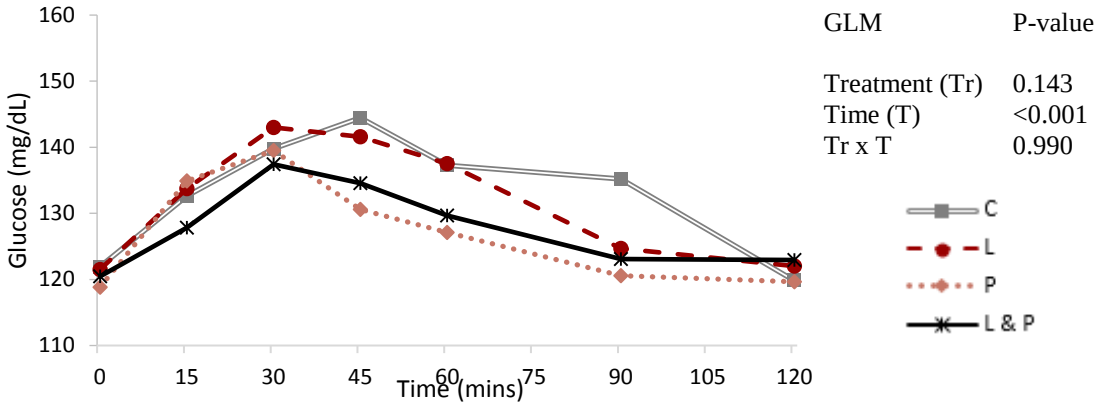
2. Glucose

The absolute glucose levels are shown in Figure 5. Even though there was a tendency for the glucose levels to be higher after the consumption of the control bread, there was no significant difference observed between the treatments (p=0.143). Moreover, results did not demonstrate a significant difference in the interaction between treatment and time (p = 0.990). The glucose levels peaked at minute 30 for the lysine, phosphorus, and the lysine x phosphorus treatments, but peaked at minute 45 for the control bread.

The change in glucose levels from zero are also illustrated in figure 6. It is worth mentioning that the change in glucose was higher in the control bread (13.27 ± 6.20

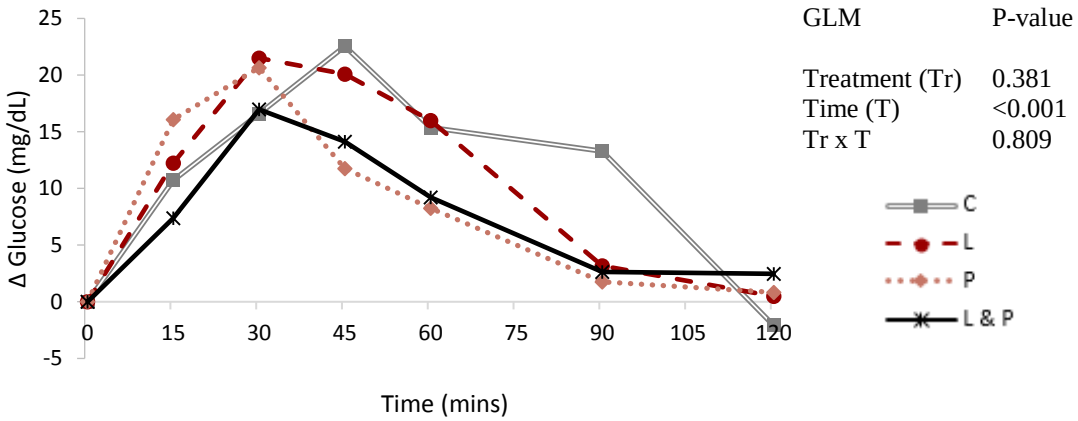
mg/dL) than the LP bread (2.64 ± 4.02 mg/dL) at minute 90. This difference almost reached statistical significance (p=0.06). Moreover, the highest mean AUC was observed with the control bread (1438 ± 507) and the lowest was with the lysine and phosphorus bread (901 ± 341), yet there was no significant difference between the different treatments (p=0.717) (Table 2, Figure 7).

Figure 5 Absolute glucose levels for the different treatments over 120 minutes



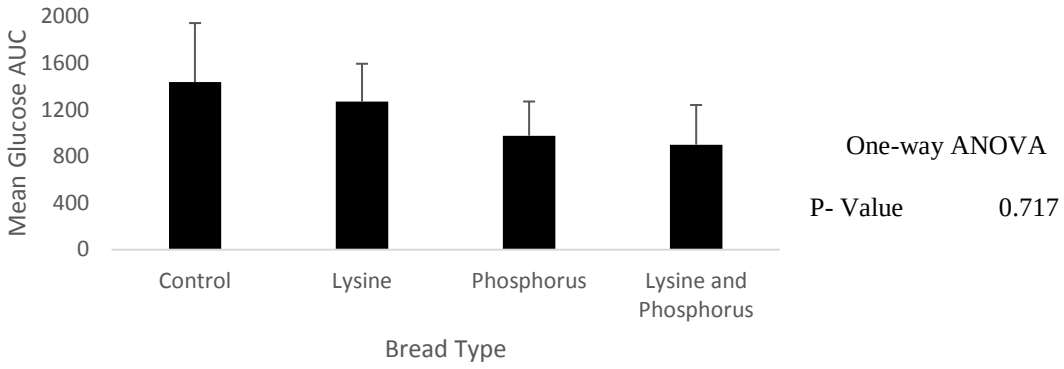
Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 6 Change in glucose levels from zero for different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 7 Mean area under the curve of Glucose for different treatments



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

3. GLP-1

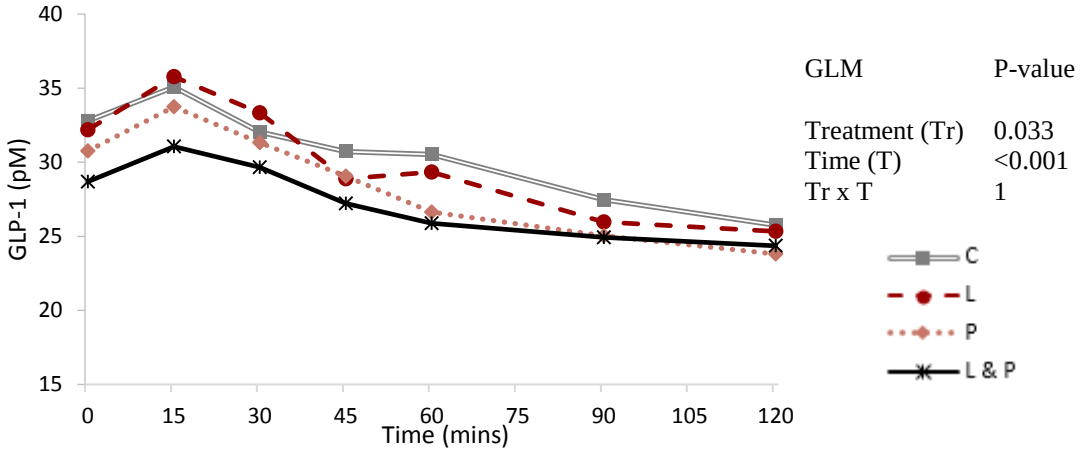
The absolute values of GLP-1 are shown in figure 8. A significant difference was observed between treatments (p=0.033) and there was also a strong time effect (p<0.001). However, no significant difference was seen in the interaction between treatment and time (p=1). Also, the mean AUC for GLP-1 did not differ significantly between the different treatments (p=0.946) (Table 2, Figure 10).

Glp-1 levels following the consumption of the control bread were higher than the ones following the consumption of the LP bread. This difference almost reached statistical significance (p=0.054). Glp-1 levels following the consumption of the Lysine bread were also higher than ones of the LP bread, but those ones were not significantly different (p=0.210)|. GLP levels significantly increased after the consumption of all 4 types of bread and started to decrease after minute 15 (p<0.001) (Figure 8).

The postprandial changes in GLP-1 levels from zero are also illustrated in figure 9. Results did not demonstrate a significant difference between treatments (p=0.718). They did, however, show a strong time effect (p<0.001). Moreover, the treatment x time

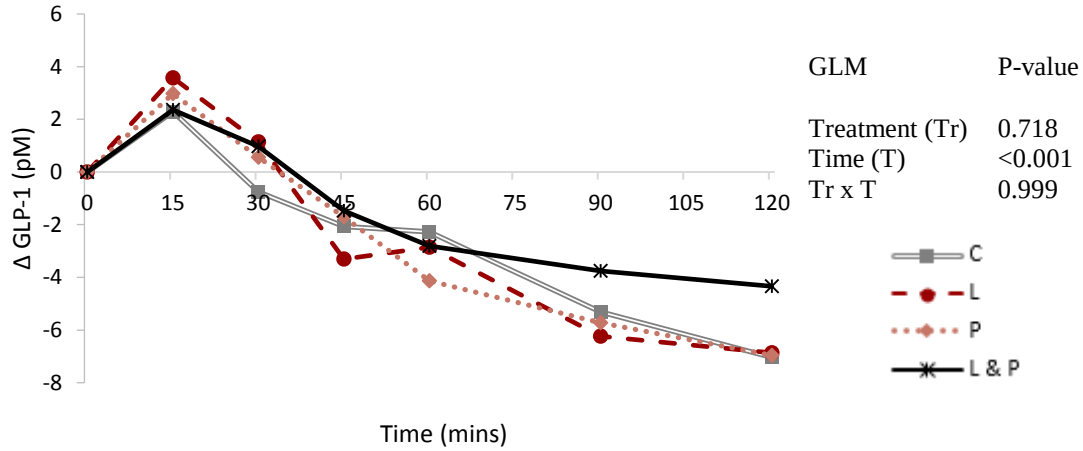
interaction did not show a significant difference (p =0.999). The highest peak was observed with the lysine bread at minute 15 (3.58 ± 1.65 pM).

Figure 8 Absolute GLP-1 levels for the different treatments over 120 minutes



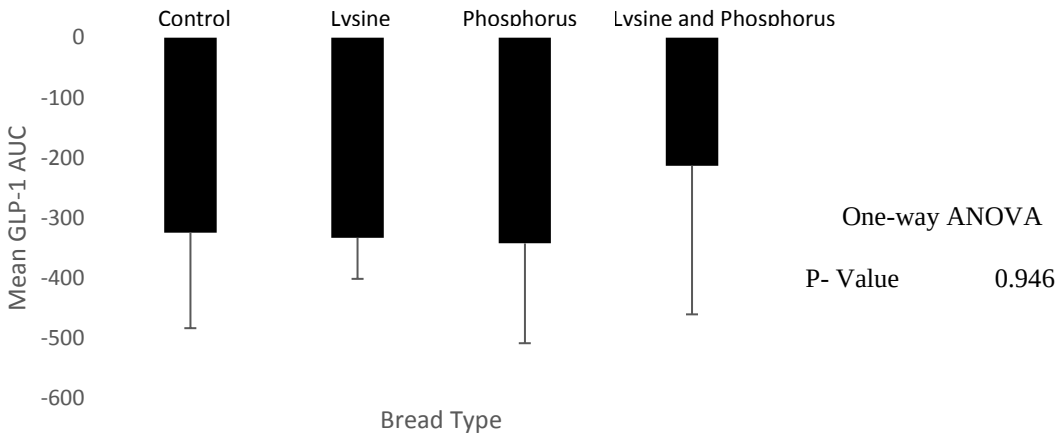
Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 9 Change in GLP-1 levels from zero for different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 10 Mean area under the curve of GLP-1 for different treatments



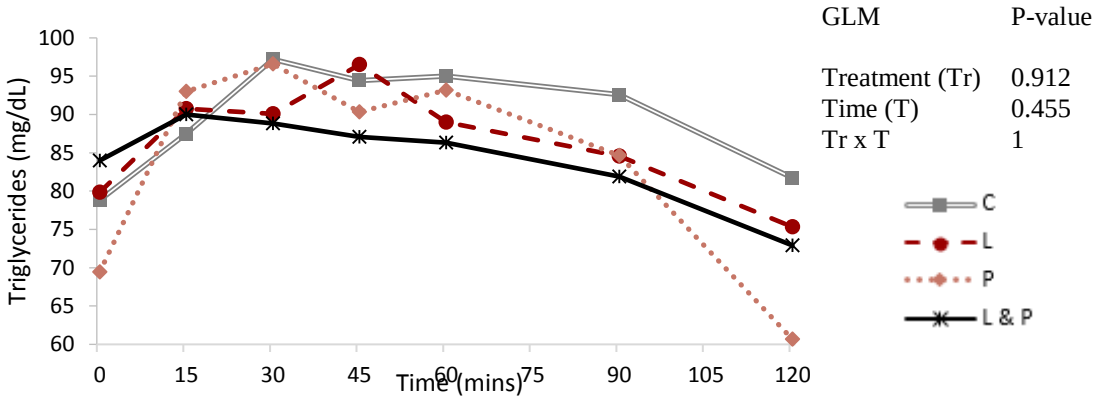
Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

4. Triglycerides

The absolute triglyceride levels are shown in figure 11. Results demonstrated showed that there was no difference between the treatments (p=0.912), and that there was no effect of time (p=0.455). Results further showed that there is no difference with treatment x time interaction (p=1). Even though the mean AUC for triglycerides was the lowest for the lysine and phosphorus bread when compared with the rest of the breads, there was no statistical significance (p=0.648) (Table 2, Figure 13).

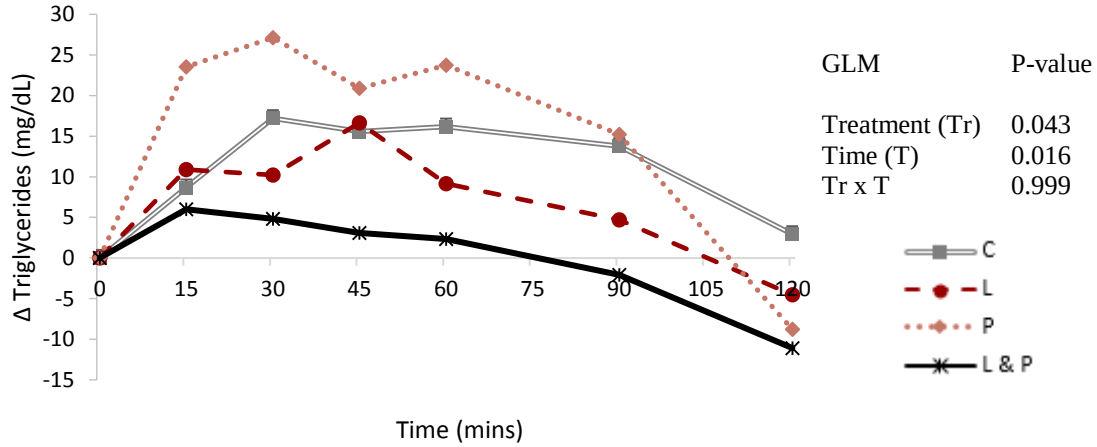
Results as a change from zero are shown in figure 12. Interestingly, there was a significant effect between treatments ($p < 0.05$) and an effect of time ($p = 0.016$). However, when the interaction between treatment and time was considered, there was no difference ($p = 0.999$). Results showed that there was a significant difference between the change in triglyceride levels after the consumption of the phosphorus bread and the change in triglyceride levels after the consumption of the LP bread ($p = 0.040$).

Figure 11 Absolute triglyceride levels for the different treatments over 120 minutes



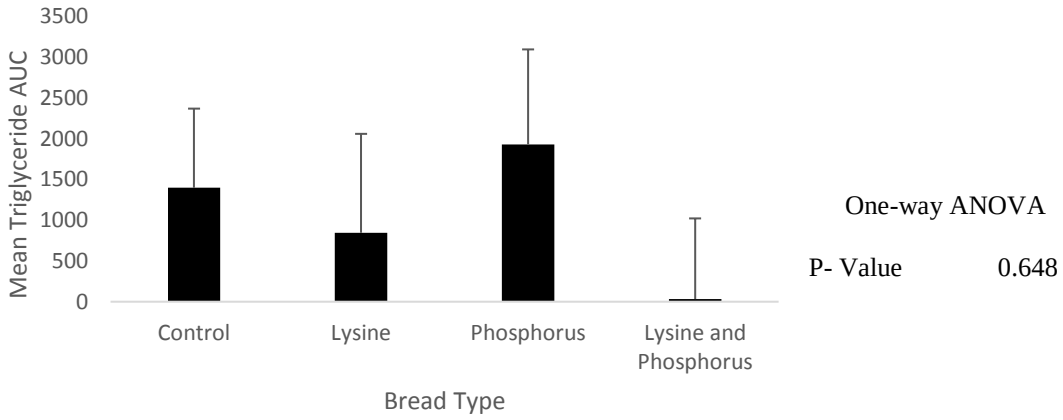
Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 12 Change in triglyceride levels from zero for different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 13 Mean area under the curve of Triglycerides for different treatments



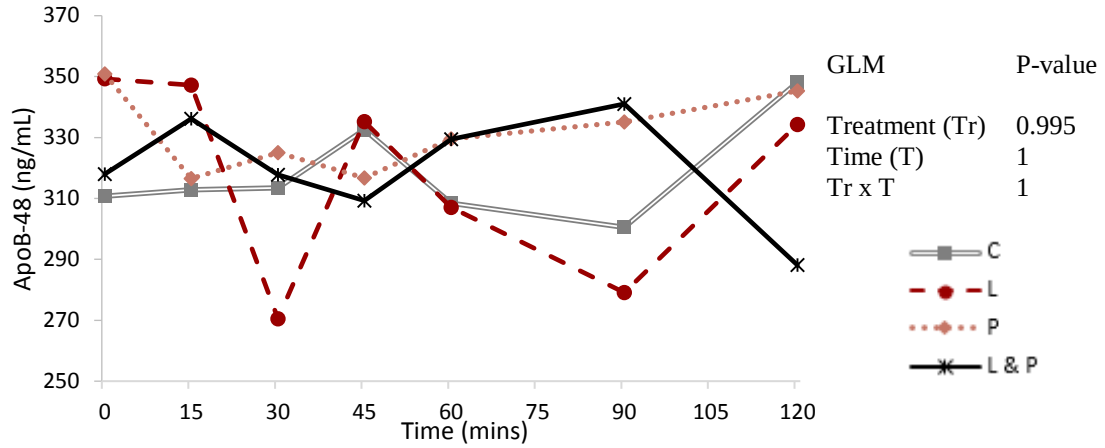
Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

5. ApoB-48

The results of the absolute Apo-B48 concentrations are shown in figure 14. There was no significant difference between the treatments (p=0.995) or an effect of time (p=1). Moreover, there was no effect of the interaction between treatment and time (p=1). The postprandial ApoB-48 changes from zero are shown in figure 15. Interestingly, there was a significant difference between treatments (p=0.005). However, there was no effect of time (p=0.844) and no significant difference between the interaction of treatment and time (0.350).

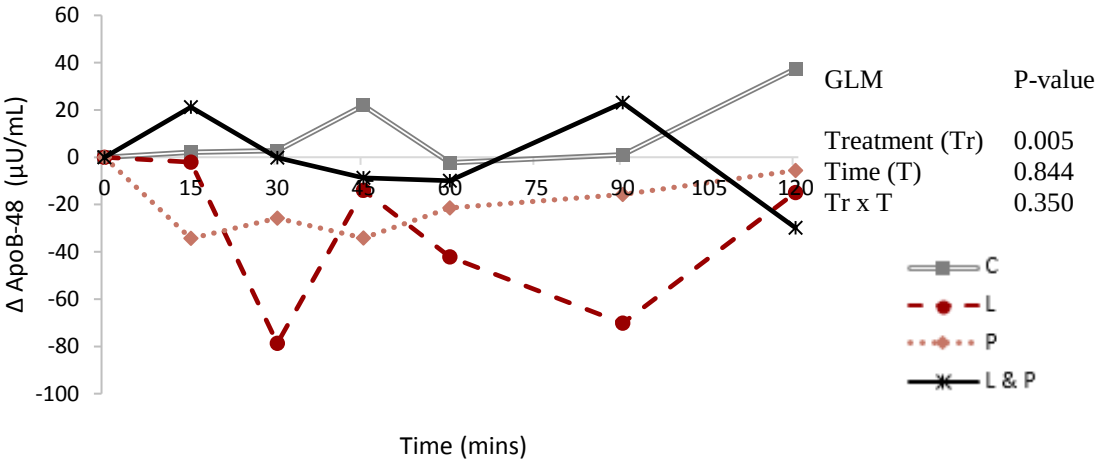
At minute 90, the ApoB-48 levels related to the lysine bread (-70.2 ± 39.8 ng/mL) were significantly lower than the ones related to the LP bread (23.0 ± 23.6 ng/mL) (p=0.007) (Figure 15).

Figure 14 Absolute ApoB-48 levels for the different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 15 Change in ApoB-48 levels from zero for different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

6. ApoB-100

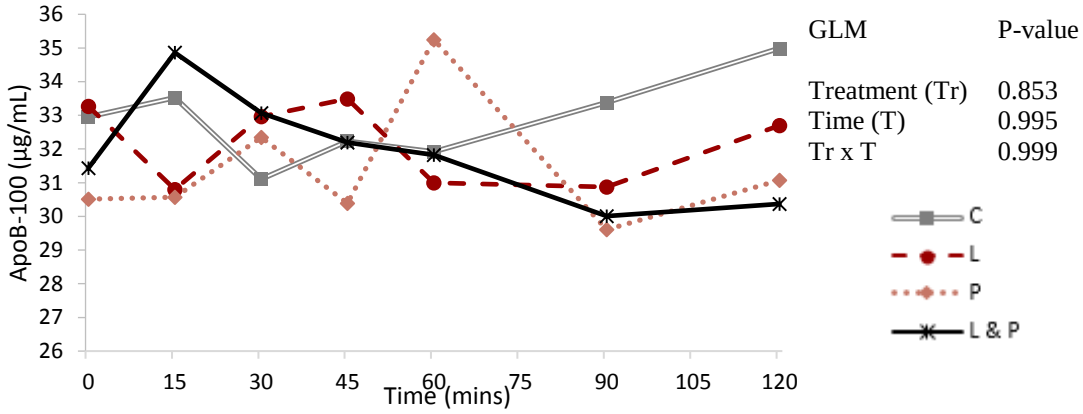
The absolute concentrations of ApoB-100 are shown in figure 16. According to the results, there was no significant difference between treatments (p=0.853) and no significant effect of time (p=0.995). Moreover, the effect of the treatment x time

interaction was not significant (p=0.999). The highest concentration of ApoB-100 was seen with the phosphorus bread at minute 60 (35.25 ± 6.38 μg/mL) (Figure 16).

Changes of ApoB-100 levels from zero are shown in figure 17. Likewise, results did not demonstrate any significant difference between the treatments (p=0.356), a significant effect of time (p=0.968), or a significant effect of the treatment x time interaction (p=0.927).

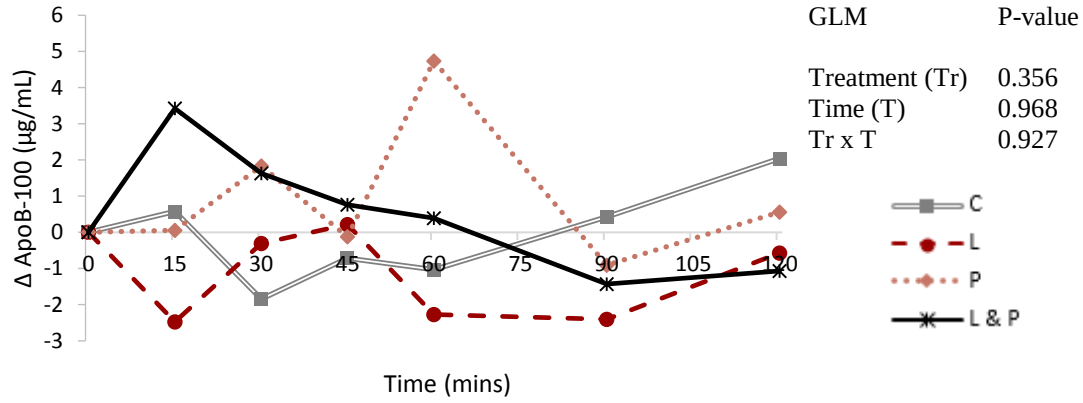
At minute 60, ApoB-100 levels with the phosphorus bread reached a maximum at 4.74 ± 5.26 μg/mL compared with the LP bread that was at 0.39 ± 2.23 μg/mL (Figure 17). This difference was just shy of reaching statistical significance (p=0.053).

Figure 16 Absolute ApoB-100 levels for the different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 17 Change in ApoB-100 levels from zero for different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

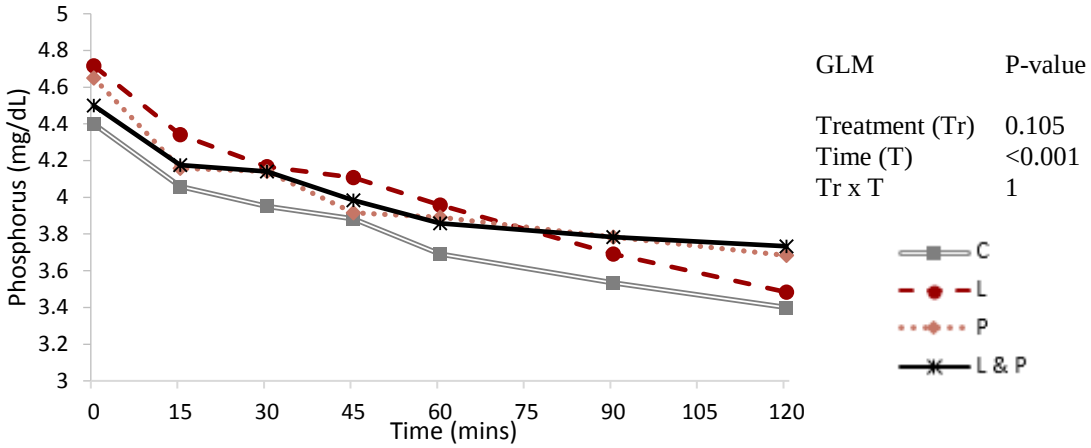
7. Phosphorus

The absolute values of phosphorus concentrations are shown in figure 18. The difference was observed between treatments was not significant (p=0.105) and no significant difference was seen in the interaction between treatment and time (p=1). However, there was a significant effect of time (p<0.001). According to figure 18,

levels of phosphorus were clearly decreasing post the ingestion of the bread, regardless of the treatment, and continued to decrease throughout the 120 minutes.

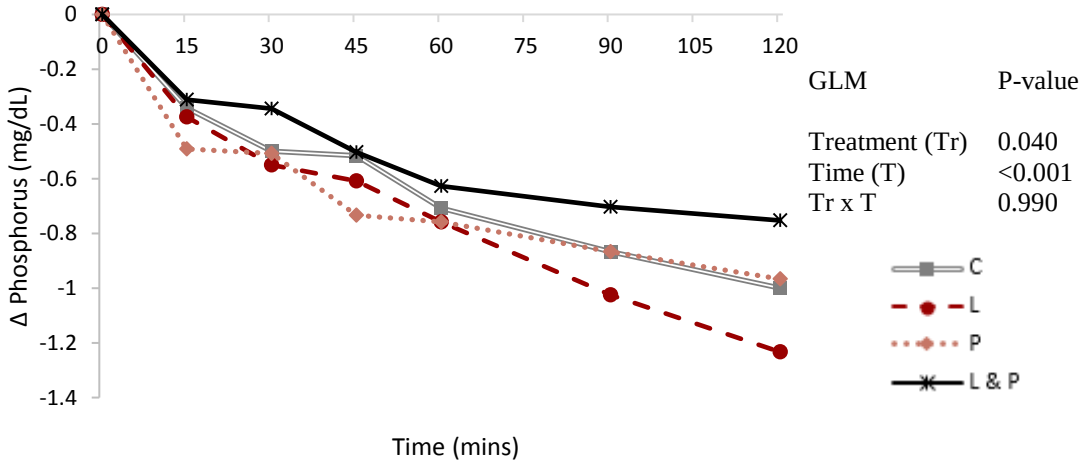
When considering the changes of phosphorus levels from baseline shown in figure 19, a difference in treatments that reached statistical significance can be seen ($p=0.04$). There was also a significant effect of time ($p<0.001$). However, the difference regarding the treatment x time interaction failed to show significance ($p=0.990$).

Figure 18 Absolute phosphorus levels for the different treatments over 120 minutes



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

Figure 19 Change in phosphorus levels from zero for different treatments over 120 minutes



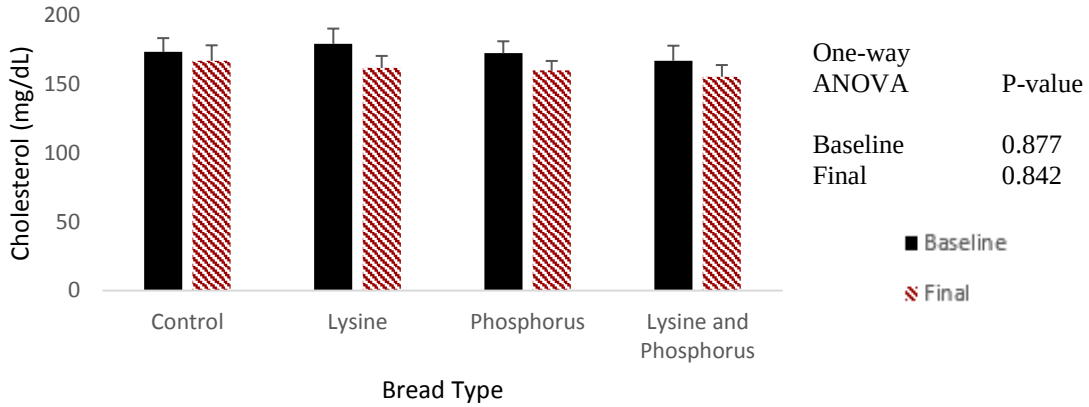
Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. General Linear Model was used, with treatment and time as the two variables, and the interaction representing treatment x time. Significance was set with P-value < 0.05.

8. Cholesterol

Results for cholesterol concentrations are shown in figure 120. The effect of treatments showed no significant difference at both the baseline measurement (minute 0, p=0.877) and the final measurement (minute 120, p=0.840). The highest change in

cholesterol levels was seen with the lysine bread (-17.50 ± 3.68 mg/dL) and the lowest change was seen with the control bread (-9.0 ± 3.17 mg/dL). Even though it is visible in the figure that cholesterol decreased between the baseline and the final measurement for all breads, this change was found to be insignificant (p=0.363) (Figure 20).

Figure 20 Cholesterol levels at minute 0 (baseline) and minute 120 (final) for different treatments

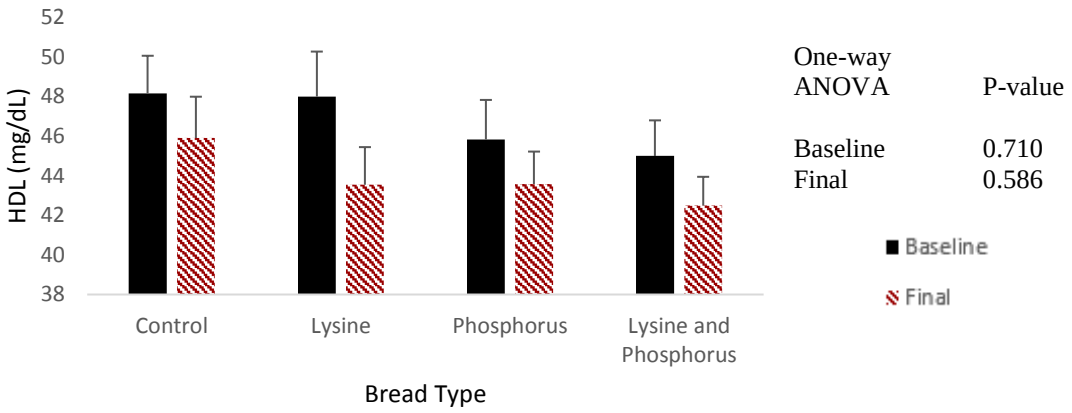


Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

9. HDL

The baseline and final concentrations of HDL are shown in figure 21. There were no significant differences in the HDL concentrations between the different bread types at baseline measurement (p=0.710) and at the final measurement (p=0.586). Regarding the change in HDL concentrations from the baseline to the final measurement, the highest change was observed with the lysine bread (-4.09 ± 1.16 mg/dL) and the lowest change was with the control bread (-2.54 ± 1.31 mg/dL) (Figure 21). However, regardless of the bread type, the change in HDL from the baseline measurement to the final measurement was deemed insignificant (p=0.615).

Figure 21 HDL levels at minute 0 (baseline) and minute 120 (final) for different treatments

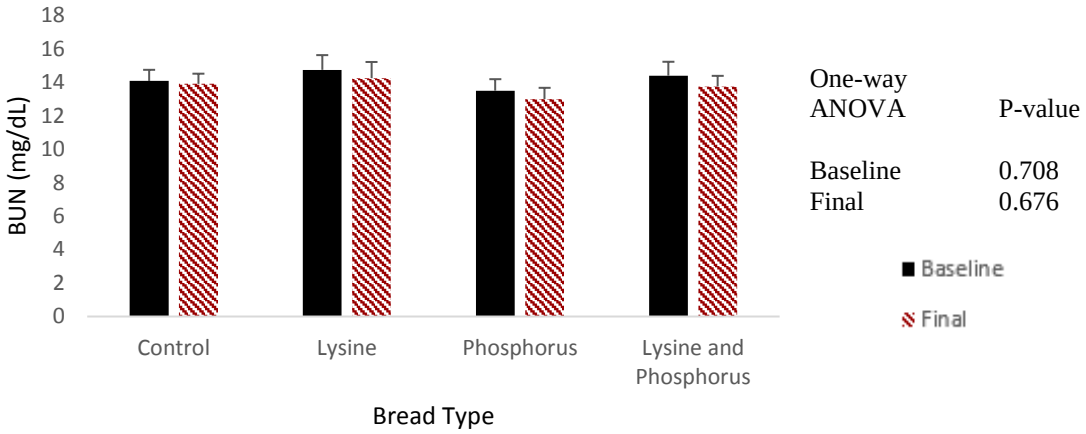


Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

10. BUN

The baseline and final BUN concentrations are shown in figure 22. The difference in treatments had no statistical significant effect on BUN levels at both the baseline measurement (p=0.708) and the final measurement (p=0.676). Change in BUN levels from baseline to minute 120 was also statistically insignificant (p=0.804).

Figure 22 BUN levels at minute 0 (baseline) and minute 120 (final) for different treatments



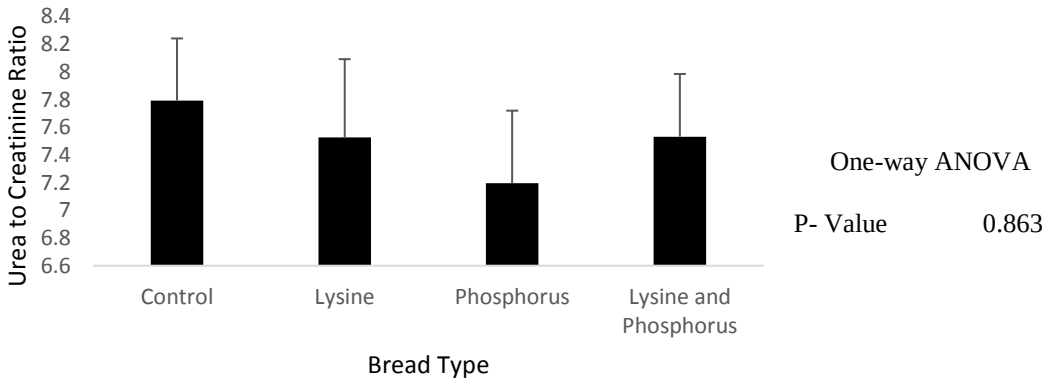
Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

C. Urine Component

1. Urea to Creatinine Ratio

The different urea to creatinine ratios with the different breads are illustrated in figure 23. The highest ratio was observed with the control bread (7.79 ± 0.45) and the lowest was with the phosphorus bread (7.20 ± 0.52). The different bread types, however, had no significant effect on the ratio of urea to creatinine (p= 0.863).

Figure 23 Urea to Creatinine levels for different treatments

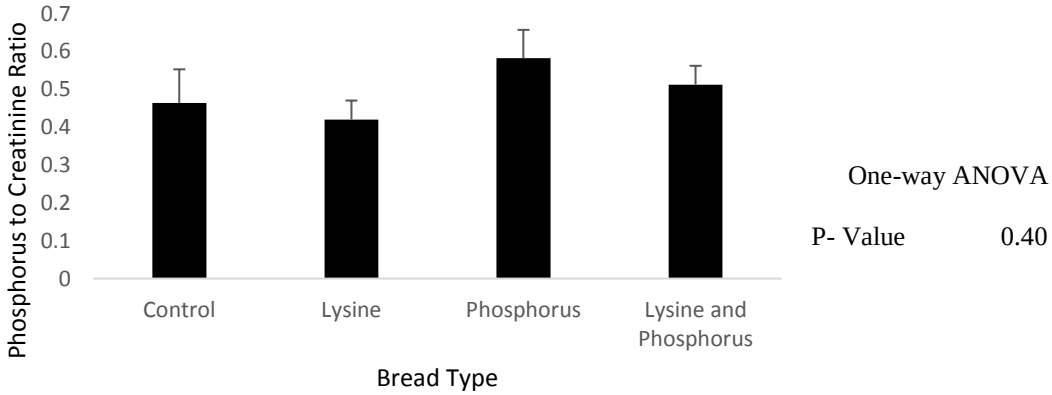


Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

2. Phosphorus to Creatinine Ratio

The different phosphorus to creatinine ratios with the different breads are illustrated in figure 24. The highest ratio was observed with the phosphorus bread (0.58 ± 0.08) and the lowest was with the lysine bread (0.42 ± 0.05). However, there was no difference between the effect of the treatments on the ratio of phosphorus to creatinine (p= 0.40).

Figure 24 Phosphorus to Creatinine levels for different treatments



Group C underwent no fortification; Group L was fortified with lysine at a rate of 0.5%; Group P was fortified with phosphorus at a rate of 0.5%; Group L & P was fortified with both phosphorus and lysine at a rate of 0.5% each. One-way ANOVA was used. Significance was set with P-value < 0.05.

CHAPTER V

DISCUSSION

Diet fortification with lysine has been associated with improvements in growth measures (Hussein et al, 2004; Ragi et al, 2018). Ragi et al. also showed that when a gluten diet was fortified with phosphorus, improvements in growth measures were observed as well. However, when they were fortified together, the effect was an estimate of a 20 fold improvement than the effect of supplementing with them separately (Ragi et al., 2018). Both lysine (Kalogeropoulou et al., 2009) and phosphorus (Khattab et al., 2015) were reported to affect postprandial glycaemia. Nevertheless, the effect of the combination of both on postprandial glycaemia and lipidemia in humans was not studied. The present study investigated the postprandial effect of bread fortified with (0.5%), lysine and 0.5% phosphorus, and the combination of both.

In the present study, the addition of lysine and/or phosphorus to bread was found to slightly reduce postprandial insulin between time 45 and 120 min. This is in line with other studies, in which intragastric infusion of lysine was reported to significantly decrease plasma insulin at 60 min compared with the control (Ullrich et al., 2017). Similarly, our study showed that insulin levels were significantly lower with the LP bread at minute 90 when compared with the control bread (Figure 2). Even though a high dose of L-Lysine monohydrochloride (621.5 mg/kg body weight) can significantly increase serum insulin (Zhang et al., 2010), our study showed that insulin levels with the LP bread were significantly lower than insulin levels with the control bread. This could be due to the fact that we did not use a high dose of lysine. On the other hand, lysine was previously shown to delay gastric emptying (Baruffol et al.,

2014), consequently slowing the release of glucose into the blood. The similarity in plasma insulin between the groups over the first 30 min argues against any impact on gastric emptying. Moreover, the similarity in postprandial plasma insulin between the lysine and/or phosphorus groups implies that no additive effect was present. On the other hand, the similarity in the changes in post-prandial glucose between the phosphorus and the phosphorus plus lysine groups implies that the changes in these groups were highly attributed to phosphorus rather than lysine.

Our study did not measure insulin sensitivity, but it is important to point out that there was a significant difference in insulin levels without having a significant difference in the levels of glucose. In other words, lower levels of insulin seen with the LP bread were able to control blood glucose similar to the higher levels of insulin seen with the control bread. This could be due to the higher levels of phosphorus found in the LP bread than those of the control. Phosphorus is needed to start auto phosphorylation, where phosphate groups get added to the intracellular part of the insulin receptor after insulin binds to the extracellular part. This sets off a cascade of reactions involving transmission of signals ending with the uptake of glucose. However, our findings contradict those of Lin et al., who reported that a low phosphorus diet enhanced insulin sensitivity in both male and female mice when compared to the control diet (Lin et al., 2018). Looking at the insulin and glucose figures as well as that of the AUC, it is clear that in the fortified groups, the reduction in the postprandial glucose was paralleled by a reduction in insulin. This implies an improvement in insulin sensitivity.

It is noteworthy that after the consumption of the LP bread, there was a trend towards a lower area under the curve for insulin and glucose when compared to the other types of bread, even though this difference did not reach statistical significance.

This was in line with the findings of Abi Rached's report, where there was a lower area under the curve for both glucose and insulin after the phosphorus preload as compared to the placebo (Abi Rached, 2012).

Even though there was a tendency for the glucose levels to be higher after the consumption of the control bread, there was no significant difference observed between the treatments. It is worth mentioning that in figure 6, one can see that glucose levels were higher in the control bread than the LP bread and remained higher, especially at minute 90 where the difference between the 2 breads almost reached statistical significance ($p=0.06$). This can be explained by different mechanisms. The LP bread was fortified with both lysine and phosphorus. A study previously demonstrated lysine's ability to delay gastric emptying (Baruffol et al., 2014). By delaying gastric emptying, lysine can possibly moderate the gradual release of glucose into the blood. Our results tie well with this as the LP bread had a lower sugar spike than the rest of the breads, even though the difference failed to reach statistical significance. Also, previous research has demonstrated the relationship between exogenous phosphorus supply and postprandial glucose. Khattab et al. showed that the co-ingestion of P with a glucose solution significantly improved postprandial glucose (Khattab et al., 2015). Another mechanism is through sugar trapping with the availability of phosphorus. Phosphate stimulates the activity of hexokinase, which is the initial enzyme of glycolysis that phosphorylates glucose to glucose-6-phosphate (Ditzel & Lervang, 2010). This explains the lower glucose levels seen with the breads that contained phosphorus when compared to the control bread.

Glucagon-like peptide-1 is an incretin hormone which strongly stimulates insulin secretion. It has a glucagon inhibitory effect and inhibits gastric acid secretion

and gastric emptying (Orskov, 1992). Our study showed that GLP-1 levels following the consumption of the control bread were almost significantly higher than the ones following the consumption of the LP bread (Figure 8). Considering that the LP and the P bread contain more phosphorus than the control bread, this contradicts a previous study that showed a phosphorus pre-load resulting in higher GLP-1 levels with the phosphorus test group when compared to the control group (Hassan, 2014). The report explained the finding by referring to how cyclic adenosine monophosphate (cAMP) is the strongest stimulus for the secretion of GLP-1 (Simpson et al., 2007). Since phosphorus plays an integral part in the formation of cAMP, they explained that an increase in its availability may lead to an increase in cAMP production, and thus increase the secretion of GLP-1 (Hassan, 2014). Even though there was a significant effect of the treatments on the absolute values of GLP-1, taking a closer look at the graph would demonstrate that there is no drastic difference between the treatments (Figure 8).

Moreover, GLP-1 levels significantly increased immediately after the consumption of all 4 types of bread and started to decrease after minute 15. This is due to GLP-1 being secreted in response to the bread. This is supported by a report which explained that GLP-1 is secreted in response to physiological stimuli e.g. a mixed meal (Orskov, 1992).

A study showed that postprandial GLP-1 response after a high protein meal was higher than a high carbohydrate meal in both lean and obese subjects (Parvaresh et al., 2018). Another study revealed that GLP-1 levels were highest after a high protein breakfast containing casein and whey (both of which are rich in lysine) when compared to high fat and high carbohydrate breakfasts (Van der Klaauw et al., 2013). Our findings

agree with these studies as the highest peak in the change of GLP-1 from baseline was observed with the lysine bread at minute 15 (Figure 9), although the difference was not statistically significant. Previously, direct stimulation of enteroendocrine cells by amino acids has been proposed as a trigger factor for GLP-1 secretion (Joshi et al., 2013). However, it remains unclear to which degree lysine plays a role when it comes to the release of GLP-1 from intact GI mucosa.

Interestingly, our results demonstrated that there was a significant difference between the change in triglyceride levels after the consumption of the phosphorus bread and the change in triglyceride levels after the consumption of the LP bread (Figure 12). Our findings contradict those of Ragi et al. which reported that the highest TG levels were found in diets that combined glucose with both lysine and phosphorus (Ragi et al., 2018). In the present study, the change in TG levels was highest after the consumption of the P bread. The difference between the P and the LP breads is the availability of lysine. One idea is that lysine is a precursor of carnitine. Since there is less lysine in the P bread than the LP bread, this could lead to a decrease in carnitine synthesis which might result in increased lipid accumulation due to less mitochondrial fatty acid oxidation. Our results were in line with the report of Bel-Serrat et al., which showed that alanine, arginine, asparaginic acid, glycine, histidine, lysine and serine intakes were inversely associated with serum triglyceride concentrations in adolescents. However, the report did point out that associations were no longer significant in both genders when total fat intake was considered as a confounding factor (Bel-Serrat et al., 2014). The opposite was shown in a study which concluded that the dietary intake of lysine was positively related to levels of triglyceride in the blood in people with spinal cord

injury. However, the authors explained that people with SCI who are known to be susceptible to dyslipidemia (Javidan et al., 2017).

Moreover, low serum phosphate levels have been associated with elevated concentrations of triglycerides (Kalaitzidis et al., 2005). In contrast, another study showed that ingesting 500mg of phosphorus after a high fat meal presented a trend towards having elevated triglyceride concentrations when compared with the 125mg P group and the control group (Hazim et al., 2014). This supports the findings of our study since the bread that was fortified with phosphorus had the highest change in triglyceride levels from baseline (Figure 12).

Usually, serum triglyceride levels are correlated with those of insulin. Insulin can activate lipoprotein lipase, which is an enzyme that plays a critical role in breaking down triglycerides (Sadur & Eckel, 1982). It is important to note that insulin resistance increases hepatic and serum triglyceride levels (Tatarczyk et al., 2011). We highlighted earlier the possible positive effect of the LP bread on insulin sensitivity. At the same time, our results showed that triglyceride levels had significantly the lowest change from baseline with the LP bread, which further supports the increased sensitivity of lipoprotein lipase to insulin with the LP bread.

The present study also demonstrated that the change in ApoB-100 levels with the phosphorus bread reached a maximum at 4.74 ± 5.26 μg/mL compared with the LP bread that was at 0.39 ± 2.23 μg/mL and this was a difference that was just shy of reaching statistical significance (Figure 17). Contradicting to our results, a study reported a decrease in apoB-100 when compared to the placebo (Hazim et al., 2014). The authors proposed that this decrease in apoB-100 possibly indicates a suppression in

hepatic production or an increase in the clearance of apoB-100 containing lipoproteins from circulation.

The breads had a significant difference in their effect on ApoB-48 levels. At minute 90, the changes in ApoB-48 levels related to the lysine bread were significantly lower than the ones related to the LP bread (Figure 15). The only difference between the two breads is the presence of phosphorus. This was in line with a study that demonstrated a significant increase in postprandial concentrations of apoB-48 after 500 mg of phosphorus with a high fat meal was ingested (Hazim et al., 2014). Apo-B48 is secreted within chylomicrons, usually in response to dietary fat. However, research has previously shown that chylomicron-apoB48 concentrations can significantly be elevated after meals with a high glycemic index, similar to ours (Parks, 2001). The author explained that high postprandial glucose and insulin concentrations may cause the liver to decrease the rate of chylomicron clearance via receptor-mediated events.

At minute 90, insulin with the control bread reached its peak and started decreasing afterwards (Figure 2). At the same time, ApoB-48 reached a dip and started increasing afterwards (Figure 14). From these results, it is clear that, insulin affected ApoB-48 levels. Also, when looking at the separate participants insulin and apoB-48 levels, two participants exhibited abnormally low insulin levels along with abnormally high apoB-48 levels. A lot of research has aimed to explain this relationship, yet resulted in different conclusions. Haas et al. explained how insulin decreases ApoB secretion by promoting ApoB degradation in the hepatocyte. The report also explained how high insulin levels act on the adipocyte to promote triglyceride uptake and inhibit free fatty acid release while low levels release free fatty acids from the adipocyte and deliver them to the liver. (Haas et al., 2013). This was further supported when a study

showed that apoB-48 and apoB-100 production rates were suppressed by 47–62% by insulin (Pavlic et al., 2010). On the other hand, insulin has been shown to decrease the production and secretion of apoB-100, but not those of apoB-48 (Allister et al., 2004). As a matter of fact, a study done on rats showed that as fasting insulin concentrations increased, the production rates of apoB-48 increased as well (Duez et al., 2006). However, research that examined the relationship between low insulin and high apoB-48 levels was limited. Increased apoB-48 levels were demonstrated in type 2 diabetic subjects, who suffered from insulin resistance, when compared to non-diabetic subjects. The authors attributed this increase to reduced fractional catabolic rates of the lipoprotein (Hogue et al., 2007).

It is important to note the lack in trend and pattern in the apoB-48 and apoB-100 levels when looking at the graphs. This could be attributed to the fact that no fat component was added to the test meal and attributed to the short post-prandial duration of blood withdrawals.

The addition of lysine and phosphorus to bread led to the least change in phosphorus levels. This can be easily explained as the LP bread had phosphorus added to it. In contrast, the P bread which was also fortified with phosphorus, was decreasing more rapidly than the rest of the treatments at different times, such as minute 15 and minute 45 (Figure 19).

The change in serum phosphate was previously related to the changes seen in insulin levels. A study showed that as soon as insulin started returning to the baseline levels, serum inorganic phosphate and total phosphate increased simultaneously (Abi Rached, 2012). Other studies explained that insulin promotes the entry of phosphate into the cells where it aids in the uptake and metabolism of glucose, which can further

lead to plasma phosphate levels falling (Wolfsdorf et al., 2009, Kritzer et al., 1956). In our study, for the P bread, we observed that when insulin was at its peak, levels of phosphorus increased briefly and then fell. We relate this falling to the phosphorus shift that moves phosphorus intracellularly where it is needed to help metabolize glucose.

Research has previously shown that dietary phosphate plays an important role in the controlling of cholesterol homeostasis by regulating gene expression in the liver (Tanaka et al., 2013). Moreover, serum phosphate has been positively associated with HDL-cholesterol (Park et al., 2008). Also, Ragi et al. showed that total cholesterol and HDL cholesterol were significantly decreased in rats with a phosphorus-fortified diet and a lysine-fortified diet (Ragi et al., 2018). This did not tie well with our results because even though there was an insignificant decrease in levels of cholesterol and HDL between the fasting and the final measurements, there was no significant difference between the treatments on neither cholesterol nor HDL levels. Interestingly, the highest decrease was seen with the lysine bread for both cholesterol and HDL. Previous experiments have assessed the effect of lysine and other essential ketogenic amino acids on cholesterol levels in the blood. However, it was lysine in combination with other amino acids, such as methionine, that had the greater effect on cholesterol levels and produced a high hypercholesterolemic response (Kurowska & Carroll, 1994; Giroux et al., 1999). These studies explained their results by mentioning that high dietary levels of lysine combined with methionine are influencing enzymes involved in the biosynthesis of phosphatidylcholine, which is the main phospholipid required for the assembly of very low density lipoproteins (VLDL). It is important to note that their results were mainly affiliated with the availability of methionine rather than lysine. Giroux et al. further showed that the various amino acid diets, including the lysine diet,

had no significant effect on HDL cholesterol concentrations (Giroux et al., 1999). Similarly, in our study, the fortification of bread with lysine did not significantly affect cholesterol levels or HDL levels. This is explainable as the amount of lysine used in our study is less than ones used before, and it was not combined with other amino acids.

BUN is used to estimate or calculate blood urea. Urea is an important metabolite of dietary protein and tissue protein turnover (Walker et al., 1990). Therefore, a decrease in BUN levels could be translated into improved protein metabolism and decreased amino acid oxidation. Hussein et al. observed an improved protein quality of the diet with lysine fortification in a population with an adequate mean total protein intake (Hussein et al., 2004). Moreover, a study showed that the mean BUN concentration significantly decreased in rats with the addition of phosphorus to the diet, but was not affected by the addition of lysine or the combination of lysine and phosphorus (Ragi et al., 2018). This is in line with a previous study that showed a decrease in protein deposition after a severe phosphorus depletion (Henry et al. 1979). Contrary to the findings of Hussein et al. and Ragi et al., our results did not show a significant difference between the effects of different treatments on BUN levels.

According to a study, creatinine excretion in urine is expected to be constant and equal to its production for individuals with normal kidney function (Heymsfield et al., 1983). Therefore, urea to creatinine and phosphorus to creatinine ratios were measured to get an accurate measurement of urea and phosphorus excretion.

High levels of urea in the urine usually indicate increased protein breakdown in the body. A review article explained that a healthy individual either processes AAs into other important metabolites or oxidizes them for energy. This oxidation results in excess nitrogen which gets disposed of as urea, the nontoxic nitrogenous waste product

(Griffin & Bradshaw, 2017). In our study, the highest ratio was observed with the control bread and the lowest was with the phosphorus bread, even though this difference was not deemed significant. It is reasonable to have the highest urea excretion with the control bread as there is not as much phosphorus or lysine to decrease amino acid breakdown.

Urinary phosphate excretion better indicates the intracellular phosphate status (Khattab et al, 2015; Hazim et al 2015). As expected, the highest excretion of phosphorus was seen with the phosphorus bread, even though the difference between the breads was insignificant. Research has shown that high dietary phosphorus intake increases serum phosphorus concentrations. This increase stimulates parathyroid hormone secretion and fibroblast growth factor-23 secretion, both of which increase phosphorus excretion in urine (Takeda et al., 2014).

To our knowledge, this is the first study to look at the postprandial effect of bread that has been fortified with both phosphorus and lysine. One of the limitations of the study is the small sample size, as a larger size would have provided more strength to the results and possibly, more statistical significance. Another limitation includes the assessment of the post-prandial effects in males only and thus, any gender related differences may have been overlooked. One major limitation is that the retention of lysine and phosphorus post-baking were not measured. This measurement would have better indicated how much of the added quantity was retained and actually consumed.

CHAPTER VI
CONCLUSION

In conclusion, the present findings demonstrated that bread fortification with phosphorus and lysine does have an effect on post-prandial glycaemia and lipidemia. Significantly lower levels of insulin were seen in the LP bread paralleled by a reduction in glucose levels. This implies better insulin sensitivity with the lysine and phosphorus fortified groups which could possibly propose a solution for those suffering from impaired glucose tolerance or insulin resistance. Also, fortification with phosphorus and lysine was able to exert a significant effect on triglyceride levels. However, postprandial changes can only be used as a starting point to hypothesize how TG production and clearance rates may be affected by differences in glycemic index.

It is highly recommended that future experiments carry a larger sample size, which possibly could add more strength to the results, particularly in trends where significance was not reached, as well as performing the same study on females to account for possible gender differences. Future work should also consider measuring insulin sensitivity to have better information about the action of insulin. Considering post-prandial lipid profile, blood withdrawals over a longer period would probably give better insight. Finally, it would be interesting to observe the long-term effects of bread fortification on glycaemia and lipidemia.

APPENDIX I

Consent to participate in a research study

Title of research study: The effect of bread fortification with phosphorus and lysine on postprandial glycaemia and thermogenesis

Specific aim 2: Determining the glycemic response after ingestion of the fortified breads

Principal Investigator: Dr. Omar Obeid/ Department of Nutrition and Food Science/ Faculty of Agricultural and Food Sciences/ American University of Beirut

Address: American University of Beirut, Hamra, Beirut, Lebanon/ 01–350000 ext 4440

Site where the study will be conducted: AUB – FAFS – NFSC department

You are being asked to participate in a research study. Before agreeing to participate in this research, it is important that you read the following information carefully. This statement describes the purpose, procedures, benefits, risks and discomforts, and precautions of the study. Also mentioned is your right to withdraw from the study at any time. You should feel free to ask any questions that you may have.

A. Purpose of the research:

We ask you to participate in this research study about effect of bread fortification with phosphorus and lysine. Wheat and wheat-derived products are highly consumed around the world. However, wheat protein are of low quality and wheat based diets can't sustain optimal growth, and accordingly should be supplemented with lysine. In addition, phosphorus is an essential mineral naturally present in our foods and required by our bodies for normal function. Phosphorus is known to be involved in energy metabolism and protein synthesis. Indeed a recent study showed improvement in energy metabolism with the addition of the combination of lysine and phosphorus to a wheat based diet.

B. Project/Procedures Description:

The total number of participants enrolled is 12, they will be recruited by direct approach on campus or by personal contact for people that might be interested from previous aim. You will be recruited to determine the postprandial glycemic response after consumption of different breads fortified with lysine, phosphorus, or lysine and phosphorus.

After agreeing on your participation, you will be asked to fill a questionnaire about general health information (Annex 3) and a blood test will be done in order to make sure that you have normal kidney functioning. In addition, a small finger pricks will be done to measure HBA1C to make sure that you have normal glucose levels. Once you fit these inclusion criteria, you will continue the study. In the case of abnormal values, you will be asked to contact your doctor, and will be excluded from the study.

You will be asked to undertake 4 visits over 4 different days following an overnight fast (>8 hours), the difference between the visits being the type of bread to be consumed. You should maintain your regular dietary and physical activity habits during the study course and avoid alcohol consumption and any unusual vigorous exercise 24 hours prior to the study. Anthropometric measurements (height, weight) will be taken and you will be asked to empty your bladder. In every visit, you will be given 50 g of bread to eat with 250 ml of water. An intravenous catheter will be inserted, by a trained practitioner into an antecubital vein for multiple blood collections. Seven blood samples will be withdrawn at different time intervals post-ingestion (0, 15, 30, 45, 60, 90 and 120 minutes) for glucose determination. In addition, mineral and several metabolites content

of the blood will be measured (glucose, total phosphorus, triglycerides, insulin and GLP-1). A urine sample will be collected right after each visit to test for urea nitrogen, creatinine and phosphorus.

Exclusion criteria: Subjects without Health Insurance Plan (HIP)**.** Any significant medical diseases (such as Diabetes, cardiovascular, cerebrovascular, pulmonary, hepatic, renal, endocrinological (PTH)), pregnancy or lactation, regular use of medication that affects body weight, a weight loss of 3% or more in the preceding 3 months. Abnormal results after the blood test and finger prick tests at baseline.

C. Duration:

The study will consist of 4 visits over 4 different days separated by one week at least, in the morning after an overnight fast. Each visit will be around 2½ hour long.

D. Benefits:

There is no direct benefit for you, but your participation will help assess the effect of lysine and phosphorus bread fortification on blood glucose and the glycemic status and lead to promising results in the control of diabetes.

Risks and Discomforts:

Your participation in this study involves only minimal risks. Blood sample collection can sometimes lead to slight skin reddening or minor bruising. In order to minimize this risk and also the anticipated discomfort for you, the blood will only be collected by medical staff with adequate training and experience. As sterile materials are used, there is no risk of any disease transmission. The potential side effects of the addition of phosphorus/lysine are nausea, diarrhea, or epigastric pain; from our experience the use of these doses is not associated with any of these signs. There may be unforeseeable risks. You can abstain from answering questions that may be sensitive.

E. Compensation/Incentive:

You will be paid the equivalent of 25$ for each visit to cover the cost of transport, adding up to 100$ for the 4 visits you will have to undertake. All study related tests and procedures will be covered by the research fund.

F. Confidentiality:

If you agree to participate in this research study, the information will be kept confidential. Unless required by law, only the study doctor and designee, the ethics committee and inspectors from governmental agencies will have direct access to your medical records.

To secure the confidentiality, your name and other identifiers will never be attached or used in our reports or published papers. Your privacy will be maintained in all published and written data resulting from this study. Blood samples will be kept for 3 years to meet AUB archive requirements and then all the files will be destroyed.

Please, indicate whether you agree or not to use your samples in future research studies

___ Yes, I would like to

___ No, thank you

G. Participants Rights:

Participation in this study is voluntary. You are free to leave the study at any time and for any reason without any negative consequences and penalty. Your refusal will involve no penalty or loss of benefits to which the subject is otherwise entitled and it will not affect participants' grades in addition to the relationship with AUB and AUBMC.

H. Payment for Research-related Injury:
The study is associated with minimal risk. However, there may be unforeseeable risks, and in the unlikely event of any adverse event as a result of the study, there will be no compensation to cover such expenses, in case it is not covered by a third party or governmental insurance.

I. Contact Information and Questions:

1- If you have any questions or concerns about the research you may contact: Dr. Omar Obeid, 009611350000-ext 4440; oo01@aub.edu.lb
2- If you have any questions, concerns or complaints about your rights as a participant in this research, you can contact the following office at AUB: Biomedical Institutional Review Board: 009611350000-ext 5440; irb@aub.edu.lb

Do you have any questions about the above information?
Please notify that the investigator has the right to end subject's participation in this study.

Would you like to be contacted for future research? Yes _____ No _____

Participant Consent:
I have read and understood all aspects of the research study and all my questions have been answered satisfactorily. I understand that I am free to withdraw this consent and discontinue participation in this project at any time, even after signing this form, and it will not affect my care or benefits. I know that I will receive a copy of this signed informed consent. I was given sufficient time to make a decision about participating in the study.
I voluntarily agree to be a part of this research study.

Participant Name: _____ Date: _____
Participant Signature: _____

Investigator's Statement:
I have reviewed, in detail, the informed consent document for this research study with _____ (name of patient, legal representative, or parent/guardian) the purpose of the study and its risks and benefits. I have answered to all the patient's questions clearly. I will inform the participant in case of any changes to the research study.

_____ _____ _____
Name of Investigator or designee Signature Date & Time

APPENDIX II

Health Questionnaire

Name: **Subject number:**

Date:
Height: _____ **Weight: _____**
(Both filled by the investigator after taking the measurements)

Please answer the following questions:

1. **Do you suffer from one or more of the following?**

Diabetes	□ No	□ Yes
Heart diseases	□ No	□ Yes
Pulmonary disease	□ No	□ Yes
Kidney problems	□ No	□ Yes
Hepatic disorders	□ No	□ Yes
Dyslipidemia	□ No	□ Yes
Hypertension	□ No	□ Yes
Cerebrovascular disease	□ No	□ Yes

Others: _____

2. **Did you undergo any surgery in the last 5 years?**
□ No □ Yes (specify :_____)

3. **Did you lose more than 3 Kilograms in the last 3 months?**
□ No □ Yes

4. **Are you currently taking any medication?**
□ No □ Yes (specify :_____)

5. **Are you a smoker?**
□ No □ Yes (specify number of cigarettes per day :_____)

6. **Do you drink alcohol?**
□ No □ Yes (specify average number of drinks per week _____)

7. **Have you been dependent on the use of drugs in the past 5 years?**
□ No □ Yes

8. **Do you take any nutritional supplement?**
□ No □ Yes (please specify _____)

9. Do you do any exercise?

□ No □ Yes (please specify type, duration and frequency_____)

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