AMERICAN UNIVERSITY OF BEIRUT

PHOSPHORUS EFFECT ON WEIGHT GAIN IN RATS FED WHEAT GLUTEN DIETS

by
MARIE-ELIZABETH ELIAS RAGI

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Nutrition and Food Sciences
of the Faculty of Agricultural and Food Sciences
at the American University of Beirut

Beirut, Lebanon
September 2016

AMERICAN UNIVERSITY OF BEIRUT

PHOSPHORUS EFFECT ON WEIGHT GAIN IN RATS FED WHEAT GLUTEN DIETS

by

MARIE-ELIZABETH ELIAS RAGI

Approved by:

Dr Omar Obeid, Professor
Nutrition and Food Sciences — Advisor

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

Dr Hala Ghattas, Assistant Professor
Epidemiology and Population Health — Member of Committee

Date of thesis/dissertation defense: September 9, 2016

AMERICAN UNIVERSITY OF BEIRUT

THESIS, DISSERTATION, PROJECT RELEASE FORM

Student Name: RAGI MARIE ELIZABETH ELIAS
Last First Middle

☑ Master's Thesis ○ Master's Project ○ Doctoral Dissertation

☑ I authorize the American University of Beirut to: (a) reproduce hard or electronic copies of my thesis, dissertation, or project; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

☐ I authorize the American University of Beirut, **three years after the date of submitting my thesis, dissertation, or project,** to: (a) reproduce hard or electronic copies of it; (b) include such copies in the archives and digital repositories of the University; and (c) make freely available such copies to third parties for research or educational purposes.

Marie Ragi 20 September 2016
Signature Date

This form is signed when submitting the thesis, dissertation, or project to the University Libraries

ACKNOWLEDGMENTS

First and foremost, my deepest recognition goes to my advisor Prof. Omar Obeid for his professional guidance, valuable help and support.

I would also like to express my sincere gratitude to the members of my committee Dr. Imad Toufeili and Dr. Hala Ghattas for their precious time and cooperation.

I am as well extremely indebted to Carla El Mallah for giving me constant assistance and precious advice.

Special thanks to Tsolaire Sourenian and Ghada Ziade for their time and help during the experimental procedure.

And finally, I am extremely thankful to my mom, family and friends for their continuous support and encouragement.

AN ABSTRACT OF THE THESIS OF

Marie-Elizabeth Elias Ragi for Master of Science
Major: Nutrition

Title: Phosphorus effect on weight gain in rats fed wheat gluten diets

Wheat gluten is the major protein source in many developing countries. Gluten lacks some essential amino acids, primarily lysine, and, accordingly, can't foster optimal growth. For that reason, it should be complemented with a protein source containing the limiting amino acid to improve growth. Wheat is also known to contain limited amounts of available phosphorus. In a recent study on rats (Hammoud et al., 2015), the addition of phosphorus to a low complete protein diet (10% egg white protein) was reported to yield weight gain comparable to that of a normal protein diet (20%). The objective of this study was to investigate the effect of phosphorus addition to a low incomplete protein diet on growth.

After receiving approval from Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB), forty male rats, of around 220 g, were randomly divided into four groups and maintained on diets containing 10% protein in the form of wheat gluten (G) with added lysine (G+L) or phosphorus (G+P) or lysine plus phosphorus (G+L+P). Body weight and food intake were measured twice per week for 9 weeks.

Food intake varied significantly when lysine or phosphorus were added to the diet. Food intake of the lysine or phosphorus groups increased by about 15%, while that of both (lysine plus phosphorus) increased by about 45%. Weight gain and energy efficiency were significantly different according to lysine, phosphorus and their interaction lysine x phosphorus. Around 5 times improvement was seen following the addition of either lysine or phosphorus, and this was further amplified to 20 times with the combination.

In conclusion, enhanced growth following the addition of both lysine and phosphorus seems to be mainly related to efficient energy utilization rather than increased energy intake. Moreover, a combination of the missing amino acid plus phosphorus is required to improve the quality of a gluten based diet.

CONTENT

ILLUSTRATIONS

Figure

TABLES

Table

ABBREVIATIONS

°C	Degree Celsius
>	Greater than
<	Less than
-	Minus
/	Per
%	Percent
x	Times
µg	Microgram
ADA	American Dietetic association
AM	Before Noon
ANOVA	Analysis Of Variance
apo	Apolipoprotein
ATP	Adenosine Triphosphate
AUB	American University of Beirut
BUN	Body Urea Nitrogen
BW	Body Weight
CRP	C-reactive protein
d	Day
dl	Deciliter
EAR	Estimated Average Requirements
DRI	Dietary Reference Intake
EDTA	Ethylenediaminetetraacetic acid
et al	and others
FAO	Food and Agriculture Organization
g	Gram
Grp	Group
h	Hour
HDL	High Density Lipoprotein
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
IACUC	Institutional Animal Care and Use Committee
Kcal	Kilocalories
kg	Kilogram
L	Liter
LDL	Low Density Lipoprotein
mg	Milligram
mmol	Millimol
ng	Nanogram
OGTT	Oral Glucose Tolerance Test
P	Phosphorus
P	p value
RDA	Recommended Dietary Allowance
SD	Standard Deviation
TG	Triglycerides
UL	Upper Limits

UNU	United Nations University
USDA	United States Department of Agriculture
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization

CHAPTER I

INTRODUCTION

The quality of protein consumed varies considerably. Developing countries, as well as vegetarian and vegan individuals, are known to follow a plant based diet depending a great deal on cereals as a major source of protein and energy (Hussain et al., 2004; Zhao et al., 2004). Moreover, in developed societies, the consumption of plant proteins is being highly encouraged notably because of the association of animal proteins with increases in the risk of some chronic diseases (ADA, 2003; Singh et al., 2003; Sluijs et al., 2010; Teixeira et al., 2000).

However, cereal proteins, of which wheat gluten, are of low quality and lack some essential amino acids, primarily lysine (Bos et al., 2005). Therefore, they induce growth impairment when used as the main source of protein in the diet (Burns et al., 1982), and accordingly should be complemented with the missing amino acids to meet human needs (Hoffman and Favlo, 2004). It has indeed been shown that supplementation with lysine enhances the quality of wheat gluten (Scrimshaw et al., 1973) and is able to slightly improve growth by increasing weight gain and food intake (Graham et al., 1981; Hussain et al., 2004; Zhao et al., 2004).

But also, it has been well documented that wheat contains limiting amount of available phosphorus. Being in the form of phytate in plants and cereals, phosphorus bioavaibility from these sources is very low because humans and other species lack phytase, the necessary enzyme needed for digestion of phytate-phosphorus (Calvo and Tucker, 2013; Kalantar-Zadeh et al., 2010). Additionally, it was recently reported

(Hammoud et al., 2015) that the addition of phosphorus to a low complete protein diet was able to induce weight gain comparable to that of a normal protein diet.

Therefore, we conducted this study to investigate whether the addition of phosphorus to a gluten diet can improve growth similarly to lysine supplementation.

CHAPTER II

LITTERATURE REVIEW

A. Proteins

1. Overview

Proteins are macronutrients that differ molecularly from carbohydrates and fat by the presence of nitrogen in their structure. In addition to their energetic role, providing 4 Kcal/g, proteins have both structural and functional roles in the body. They serve as major constituent of muscles and tissues, and provide amino acids to help in the production of cell membrane proteins, enzymes, hormones, transport carrier proteins, immunoproteins and other components essential to maintain the normal cellular and organ function, growth and health of the body (Hoffman and Favlo, 2004).

Current recommendations (DRI report, Food and Nutrition Board, Institute of Medicine, 2002/2005) state that healthy human adults of all ages require 0.8 g of protein per kilogram of body weight per day. Protein requirements increase in certain conditions such as stress, growth, pregnancy and lactation and some disease states. Therefore, protein intakes may range from 10-15% up to 35% of total energy intake to ensure a nutritionally adequate diet for all adults.

Much focus was given to the health effects induced by the variation of protein quantity in the diet. Low dietary protein was shown to unfavorably affect growth. In fact, proteins are needed to sustain the growth of the body during the period of development, and insufficient dietary amount of this nutrient provoked a decrease in weight gain, food intake and feed efficiency in rats as reported by Mercer et al (1981). A decrease in weight gain with very low protein diets (<5% protein) was also observed

in several studies (Donald et al., 1981; Peng et al., 1974; Swick et al., 1983). An inadequate protein intake was also found to provoke the down-regulation of energy metabolism and protein synthesis (Thalacker-Mercer et al., 2007). However, the impact held by the variation of the quality of dietary proteins is also of high importance.

2. Dietary Sources

Proteins are available in a variety of dietary sources. The most important are animal foods such as meat, poultry, fish, eggs, and dairy products. They contain high levels of protein and constitute the main dietary contribution, but not the only one. Proteins can also be found, in lesser amounts, in many plant and vegetable products which include legumes, cereals, nuts, seeds, fruits, vegetables, and starchy roots (DRI, 2002/2005).

However, animal and plant sources differ not only by the quantity of protein, but also by the quality of those proteins and their amino acids availability. The quality and nutritional value of a dietary protein source is determined by its essential amino acid composition, digestibility and bioavailability (FAO/WHO/UNU, 1985). Animal proteins are termed “complete protein” because they provide all nine essential amino acids in adequate amounts and thus are of high quality and able to sustain growth, whereas proteins from plant sources tend to lack one or even more of the indispensible amino acids, like for instance lysine and threonine, making them “incomplete proteins” of inferior quality (FAO/WHO/UNU, 1985). Studies indicated that plant proteins have lower biological value, protein efficiency and net protein utilization than animal sources with a net protein utilization of about 50% for wheat gluten, compared with nearly 100% for meat, milk, and eggs (Hussain et al., 2004; Zhao et al., 2004). The nine essential amino acids have carbon skeletons that cannot be synthesized by humans and

they can only be obtained through the diet, while our body has the capacity to produce the rest. Consequently, the absence of one of them from the diet will compromise normal tissue growth and maintenance (Hoffman and Favlo; 2004).

Table 1: Comparison Between the Essential Amino Acid Composition of Major Protein Sources (adapted from DRI, 2002/2005)

Amino Acid	Sources (mg/g protein)			
	Whole wheat flour	Navy beans	Milk	Eggs
Histidine	22	28	28	24
Isoleucine	40	42	60	63
Leucine	63	76	98	88
Lysine	26	72	79	70
Methionine + cysteine	35	19	34	56
Phenylalanine + tyrosine	81	77	96	98
Threonine	27	39	45	49
Tryptophan	11	10	14	16
Valine	43	46	67	72

The sources of protein consumed around the world vary considerably. While Western and developed societies main protein sources come from animal products, with plant sources of protein contributing to a minor proportion of the intake (Singh et al., 2003); individuals in developing countries or those following a vegetarian diet greatly depend on plants as the major protein source, with cereals and notably wheat accounting for a significant part of both the protein and energy supply (Bos et al., 2005; Hussain et al., 2004; Zhao et al., 2004). That is the case of Lebanon. According to the FAO (Food and Agriculture Organization of the United Nations) food balance sheet for the year

2011, cereals contribute to about 35.5% and 33% of the total energy and protein intake respectively, while only 13.8% of the total energy was obtained from animal products. Wheat in particular was found to be the most consumed, taking up to 88.4% of the cereal intake, leading to a minimal intake of lysine.

3. Protein Complementarity

Wheat contains mostly carbohydrates and only a moderate quantity of protein (8-12% on a weight basis), with gluten as the main form constituting up to 80-85% of the total wheat protein, the rest being storage proteins. In addition to the relatively low protein content, the quality of these proteins is considered poor due to the limited amount of some essential amino acids, primarily lysine. Based on their amino acid profile, gluten proteins are called “prolamins”; in fact, they possess a high content of proline and glutamine, occurring at the expense of indispensable amino acids present in very small quantities, particularly lysine as the first limiting amino acid, and to a lesser extent threonine (Bos et al., 2005). In fact, lysine is considered the main limiting amino acid in plant proteins, as lysine intakes were found significantly lower in vegans than in omnivores (Krajčovičoá-Kudláčková et al., 2000).

As mentioned, the dietary inadequacy of one of the essential amino acids will have major detrimental impacts on our health and lead to similar signs to that of protein deficiency such as decreased food intake, loss of body weight, shortening of the life span, decreased protein synthesis and reduced amino acid oxidation (Ousterhout, 1960; Sidransky and Baba, 1960; Yamashita and Ashida, 1969). Therefore, when individuals restrict their diet to protein from plant sources, notably wheat, they will get inadequate amounts of the limiting amino acids, mainly lysine, and in consequence such diets may not be able to foster optimal growth. In a study by Burns et al. (1982), weight gain, food

intake and feed efficiency were all found higher in animal protein (casein) that two sources of plant proteins, soy and wheat gluten. Moreover, it was shown that proteins from animal sources induce higher energy expenditure than vegetable protein (Mikkelsen et al., 2000; Westerterp-Plantenga, 2003).

Accordingly, due to the lack of lysine and lower protein quality of cereals, predominantly plant-based diets must be supplemented with the limiting amino acid or complemented by another protein source with better quality or higher lysine content, such as animal protein or legumes. This combination will provide a balanced amino acid pattern, improve the quality of protein and enhance the adequacy of the diet, thus matching human needs (Hoffman and Favlo, 2004; Hussain et al., 2004; Zhao et al., 2004). Mattar and Obeid (2010) also reported that the supplementation of wheat gluten by as little as 5% casein was enough to improve the dietary value of gluten and prevent the decrease in feed efficiency occurring with plant-based diets.

Many developing countries already complement their predominantly cereal protein diet with a better-quality protein supplying enough of the limiting amino acid to increase the utilization of dietary protein to an acceptable level (Hussain et al., 2004). Unfortunately, this can be unaffordable in many countries, and the fortification of staple foods by the missing amino acid can be a possible alternative for such populations (Zhao et al., 2004). In fact, it has been shown that fortifying wheat flour with lysine can significantly induce improvements in the protein quality, nutritional value and utilization of wheat gluten (Scrimshaw et al., 1973). In addition, the growth and development (Chase et al., 1976; Graham et al., 1981; Jansen and Chase, 1976), as well as the nutritional status and immune function of individuals consuming a wheat-based diet were all enhanced by lysine fortification of wheat (Hussain et al., 2004; Zhao et al., 2004).

4. Health Impacts

The consumption of plant proteins has been highly encouraged because they displayed many health benefits compared to animal sources.

On one hand, high quality animal proteins were proven to be essential in any stage of life. They were found to yield an important role during late pregnancy. Godfrey et al. (1996) reported that low protein from meat and dairy products during that stage was associated with low birth weights in infants. Moreover, toward the late stage of life, meat-based diets lead to an increase in lean body mass in elderly compared to diets with exclusively plant proteins (Campbell et al., 1999). However, health concerns were raised over the high content of fat in animal products, mostly saturated fat and cholesterol, which was found to increase the risk of cardiovascular diseases and type 2 diabetes (Hoffman and Favlo; 2004; Krauss et al., 2000).

On the other hand, diets that emphasize a greater consumption of plant foods at the expense of animal proteins were shown to play an important role in preventing many chronic diseases such as cardiovascular diseases, dyslipidemia, type 2 diabetes and many types of cancers (ADA, 2003; Bazzano et al., 2002; Montonen et al., 2003; Potter, 2000). In fact, a very low meat intake was linked to an increase in life expectancy (Singh et al., 2003). Moreover, Sluijs et al. (2010) showed that only higher intake of total and animal protein, but not of vegetable protein, were associated with increased diabetes risk. But also, the onset of atherosclerosis was reduced in animals fed a vegetable protein diet compared to animal protein (Kritchevsky et al., 1981). Replacing animal proteins with soy also significantly lowered blood levels of total cholesterol, LDL cholesterol, and triglycerides without affecting HDL cholesterol (Fernandez et al., 1999; Krauss et al., 2000; Teixeira et al., 2000). Additionally, in many

other experimental studies on animals and humans, plasma cholesterol concentrations decreased with the consumption of vegetable protein (ADA, 2003; Nagata et al., 1998; Terpstra et al., 1991). High intakes of wheat gluten in particular had beneficial effects on cardiovascular disease risk by significantly reducing oxidized LDL, serum triacylglycerol and uric acid (Jenkins et al., 2001).

Therefore, a combination of plant proteins that supply all the essential amino acids, can provide an excellent alternative source of protein, with lower saturated fat and cholesterol than animal protein, and that help protect against many diseases and sustain growth.

B. Lysine

1. Requirements

Lysine being one of the nine indispensable amino acids that cannot be synthesized by the human body, it should essentially be provided by the diet in adequate amounts. Numerous studies focused on finding lysine requirements for humans. Depending on the method used in the studies, the lysine requirements of a healthy human adult varied and a wide range from 12 to 45 mg/kg/d was reported (El Khoury et al., 2000; FAO/WHO/UNU, 1985; Kurpad et al., 2001; Kurpad et al., 2002; Kurpad et al., 2003; Millward et al., 2002; Millward et al., 2000). Many stated that the lower value of 12 mg/kg/d set at the time as international recommendations (FAO/WHO/UNU, 1985) was underestimated. The mean requirement of 30 mg/kg/d of lysine was found suitable for healthy adults throughout the world (Kurpad et al., 2001; Kurpad et al., 2002; Young et al., 1989) or 50 mg lysine/g protein when the total protein intake meets the FAO/WHO/UNU estimated average protein requirement of 0.6 g/kg/d (El Khoury et al., 2000), and a higher value of 44 mg/kg/d was estimated for undernourished

individuals (Kurpad et al., 2003). The latest report by the FAO/WHO/UNU published in 2007 reset the international recommendations at 30 mg/kg/d of dietary lysine for a healthy human adult.

Table 2: Essential Amino Acid Requirements (FAO/WHO/UNU, 2007) and RDAs (DRI, 2002/2005)

Amino acid	Requirements for adults (mg/kg/d)	RDA for adult >19y (mg/kg/d)
histidine	10 mg/kg/d	14 mg/kg/d
isoleucine	20 mg/kg/d	19 mg/kg/d
leucine	39 mg/kg/d	42 mg/kg/d
lysine	30 mg/kg/d	38 mg/kg/d
methionine + cysteine	15 mg/kg/d	19 mg/kg/d
phenylalanine + tyrosine	25 mg/kg/d	33 mg/kg/d
threonine	15 mg/kg/d	20 mg/kg/d
tryptophan	4 mg/kg/d	5 mg/kg/d
valine	26 mg/kg/d	4 mg/kg/d

It was reported that lysine being an essential amino acid, the presence of an adequate amount in the diet is necessary for growth and development. But, dietary lysine is also needed for maintenance of body weight, although the requirements during the maintenance period are lower than those of the growth stage (Millward et al., 1997; Neuberger et al., 1945). Therefore, age-related requirements for lysine are suggested, with higher values for infants as compared to adults, for instance 64 mg/kg/day are recommended for 0.5 year old infants. Moreover, requirements also change depending

on the physiological state of the individual, where they are set to increase during pregnancy and lactation (Tomé and Bos, 2007).

Concerning the needs of growing rats, a study (Stockland et al., 1970) determined that the lysine requirements for maximum weight gain are 0.6% of a diet containing an equivalent of 10% of dietary protein. Moreover, lysine needs for maximum growth were found higher with the increase of wheat gluten quantity in the diet, when this protein is the exclusive source of dietary lysine. They increased from 0.9% for a diet containing 30% wheat gluten to about 1.2% for a diet with 60% of wheat gluten (Munaver and Harper, 1959).

2. Lysine and Growth

It has been repeatedly shown in the literature that an adequate dietary amount of dietary lysine is necessary for growth and development. Mattar and Obeid (2010) found that the increase in the contribution of wheat gluten to the overall dietary protein supply, leading to a minimal lysine intake, will induce a decrease in total body weight, weight gain, food intake and feed efficiency. In fact, lysine deficiency, a common occurrence when plant proteins constitute the only source of protein in the diet, was extensively reported to provoke growth retardation and decrease food intake, and this was reversed by the introduction of lysine to the diet (Graham et al., 1981; Harper et al., 1955; Munaver and Harper, 1959; Yamashita and Ashida, 1969). A study by Millet and Aluwe (2014) found that diets restricted in lysine decreased performance, and reduced daily weight gain and feed efficiency, while both were enhanced with high lysine diets. Moreover, growth, weight gain, food intake and feed efficiency all improved with the increase of dietary lysine amounts in other species (Guzman et al, 2013; Li et al., 2014; Walton et al., 1984). In rats, Chang and Chao (1969) showed that body weight increased

as the level of added dietary lysine increased, reaching a maximum with 0.4% of lysine in the diet; but liver fat was reduced with the presence of lysine.

However, the supplementation of plant-based diets with lysine alone was not always sufficient to produce major improvements in weight, and the addition of threonine was found necessary to reverse growth retardation (Howe and Dooley, 1963; Tanphaichitr et al., 1976). Moreover, lysine fortification of wheat flour was able to just slightly increased weight and height of children (Hussain et al., 2004; Zhao et al., 2004).

3. Health Impacts

The increase of the quantity of lysine in the diet was also linked to many health manifestations, such as the reduction of lipid deposition in tissues (Hlais et al., 2012; Kroeckel et al., 2013). In fact, an important role of lysine is its involvement in the hepatic synthesis of carnitine. Lysine is a precursor of carnitine, which is required for the intra-mitochondrial transport of long-chain fatty-acyl groups for beta-oxidation. Dietary lysine deficiency in rats was found to reduce carnitine levels in the body, consequently reducing the capacity of fatty acid oxidation and increasing lipid accumulation, notably triglycerides and total cholesterol, in many tissues (liver, heart muscle). All of those were corrected by the supplementation with lysine (Harper et al., 1955; Khan and Bamji, 1979; Tanphaichitr et al., 1976) but not by that of carnitine (Tanphaichitr et al., 1976). Plant proteins constitute a minor source of carnitine in the diet because of their lack of lysine, while the main dietary contribution comes from animal products rich in lysine. In a typical omnivorous diet, it was estimated that around 75% of carnitine is provided from the diet while only 25% comes from the de novo biosynthesis from its precursor lysine (Tein et al., 1996). Therefore, populations consuming predominantly wheat-based diets are at risk of carnitine deficiency.

Lysine also exerted a role in glycemic control. It improved insulin sensitivity by increasing postprandial glucose clearance without affecting insulin release (Hlais et al., 2012; Obeid et al., 2008; Sulochana et al., 2002). In addition, lysine was reported to induce an increase in the levels of albumin (Scrimshaw et al., 1973) and pre-albumin (Zhao et al., 2004).

C. Phosphorus

1. Generalities

Phosphorus is a critical component of living organisms required by all body cells for normal functioning, and an essential mineral that plays important roles in several key biological processes. It is the sixth most abundant mineral in the body, and the second of the body inorganic elements, with a total content in the average adult of about 700 g. Phosphorus is not proportionally distributed in the body, with the majority constituting 85% found in the skeleton (bones and teeth), 14% in soft tissues and muscles, and only 1% in extracellular fluid. Normal serum concentration is 2.5–4.5 mg/dl (0.80–1.45 mmol/l) (Shills et al., 2005).

Phosphorus is directly involved in ATP generation and energy production through carbohydrate, protein and fat metabolisms. It is highly needed for skeleton mineralization, being part of the bone apatite. Phosphorus is also a major constituent of many cellular compounds such as phospholipids, nucleic acids and nucleoproteins. In addition, many hormones, enzymes, and intracellular signaling molecules depend on phosphorus for activity. Therefore, an adequate dietary intake of phosphorus is necessary to maintain structural and functional welfare of the body. Both phosphorus deficiency and excess can have detrimental effects on health and several organs normal functioning and affect the production of ATP, synthesis of nucleic acids, and formation

of cell membranes, thus causing anorexia and reduced growth but an excessive amount was found to have deleterious effects on bone (Amanzadeh and Reilly, 2006; Hazim et al., 2014; Kalanter-Zadeh et al., 2010; Obeid et al., 2014; Takeda et al., 2014; Tanaka et al., 2013).

The Recommended Dietary Allowance (RDA) of phosphorus for healthy adult men and women (19 yrs and older) have been fixed to 700 mg/day (Institute of Medicine, 1997). However, higher RDAs are required during growth periods of late childhood and adolescence. It was claimed (Obeid, 2013) that those recommendations are based on the lower limit of serum inorganic phosphorus in adults, and if taking the middle range the RDAs would be greater and better at covering our needs. However, till now no modifications have been done.

Table 3: Phosphorus Recommendations for Men and Women (Institute of Medicine, 1997)

Age (years)	RDA (mg)	EAR (mg)	UL (mg)
4–8	500	405	3000
9–13	1250	1055	4000
14–18	1250	1055	4000
19–30	700	580	4000
31–50	700	580	4000
51–70	700	580	4000
71+	700	580	3000

Dietary phosphorus is well absorbed along the entire intestinal tract, with two pathways of intestinal absorption observed. Passive paracellular diffusion is predominant when inorganic phosphorus concentration is high in the lumen. However,

when phosphorus dietary intake and concentration are low, the active transport via luminal sodium phosphorus co-transporters (primarily regulated by 1,25-dihydroxyvitamin D [1,25(OH)2D]) is stimulated to increase phosphorus absorption and utilization. The rate of absorption is highly variable, depending on the dietary sources of phosphorus. Phosphorus excretion is mainly urinary, with the kidneys, and to a lesser extent the small intestine, responsible in maintaining phosphorus homeostasis (Shills et al., 2005).

2. Dietary Sources

Phosphorus is abundantly supplied in the diet. Organic phosphorus is naturally present in most foods and related to the high protein content (Table 4). It is richest in animal-based foods like dairy products, meats, poultry, fish and eggs; but also found in plant sources of proteins such as cereals, grains, legumes and nuts. However, other significant yet incontrollable sources of phosphorus in western diets, contributing to more than 30% of phosphorus intake, were attributed to processed goods and fast foods (Calvo and Tucker, 2013; Kalantar-Zadeh et al., 2010; Uribarri and Calvo, 2013). Indeed, inorganic phosphates are widely used in food processing as additives and preservatives to enhance appearance and shelf life, and support a variety of other functions (Anderson, 2013; Calvo and Uribarri, 2013; Takeda et al., 2014). And with the growing consumption of processed conveniences, fast foods, soft drinks, restaurant meals and high protein diets in many western countries, phosphorus intake is increasing and exceeding the recommended amount of 700 mg/d (Amanzadeh and Reilly, 2006; Calvo and Tucker, 2013; Takeda et al., 2014; Uribarri and Calvo, 2013).

Phosphorus absorption is the most efficient from the sources with added phosphorus during processing than from those with phosphorus a natural component of

foods. Indeed, inorganic phosphorus salts from additives are highly bioavailable, they are not bound to protein thus are more readily and almost completely absorbed in the intestinal tract. It is believed that inorganic phosphorus from additives is >90% absorbed in the gut in contrast with only 40 to 60% of organic dietary phosphorus naturally present in food (Kalantar-Zadeh et al., 2010). However, digestibility of animal-derived phosphorus is higher than that from plant proteins (Calvo and Tucker, 2013; Takeda et al., 2014; Uribarri and Calvo, 2013). Organic phosphorus in animal sources present in intracellular compartments is easily hydrolyzed and rapidly absorbed, while in plants such as grains and legumes, phosphorus is principally stored in the form of phytate (Table 5). It is well known that enzymatic cleavage by phytase is needed to liberate phosphorus before absorption; however, the absence of phytase in humans and many mammals reduces considerably the digestibility and bioavailability of phosphorus coming from plant sources (Calvo and Tucker, 2013; Kalantar-Zadeh et al., 2010). Indeed, plant-based vegetarian diets led to lower phosphorus serum levels and absorption compared with meat based diets (Moe et al., 2011). But also, adding phytase to diets was found to induce an in increase phosphorus plasma concentration and digestibility (Kumar et al., 2010; Symeou et al., 2014). Furthermore, it was reported that the bioavailability and content of plant-derived phosphorus are influenced by food preparation methods, since for instance boiling and soaking of legumes was found to remove phytate, and the refinement of cereals reduced the amount of phosphorus by about 70% (Calvo and Uribarri, 2013; Kalantar-Zadeh et al., 2010). Moreover, malting and 12h fermentation were proven to be the most effective in reducing phytic acid and increasing non phytate phosphorus in sourdough (Mahgoub and Elhag, 1997). Yeast, used for fermentation, contains phosphatase capable of hydrolyzing phytate, hence doubling yeast and prolonging fermentation time was found to increase inorganic

phosphorus in bread (Harland and Harland, 1980). However, the contribution of yeast to phytate degradation was found very small. Yeast fermentation for up to 4h led to the breakdown of only 10% of phytic acid, while a prolonged fermentation of 5h reduced phytate by 38 % and increased soluble phosphorus by only 5% in whole wheat bread (Lopez et al, 2001).

Hence, despite the apparent high content of phosphorus in plants, they are not considered good sources because of the low intestinal absorption and bioavailability.

Table 4: Phosphorus and Protein Content of Some Animal and Plant Food Items

	Phosphorus (mg/100g)	Protein (g/100g)	P-to-Protein ratio (mg/g)
Animal-based food			
Ground beef, cooked	222	26	8.5
Chicken breast	246	28	8.8
Tuna, canned in water	216	23	9.4
Whole egg, 1 large	140	11	12.7
Milk, whole	84	3	28
Hard cheese	511	25	20.4
Cottage cheese	150	10	15
Plant-based food			
Wheat flour	108	10	10.8
Brown rice, cooked	77	2	38.5
Pasta, cooked	58	6	9.6
Soybeans	245	18	13.6
Black beans, cooked	142	9	15.8
Lentils, cooked	180	9	20
Peanuts	345	27	12.8
Broccoli, cooked	67	2	33.5

(Adapted from "U.S. Department of Agriculture, Agricultural Research Service.2016. USDANational Nutrient Database for Standard Reference, Release 28. Nutrient List Home Page,http://ndb.nal.usda.gov/ndb/nutrients/index")

Table 5: Total and Phytate Phosphorus Content of Some Plant-Based Food Items (adapted from Ravindran et al., 1994)

	Total P (g/100g)	Phytate-P (g/100g)	Phytate-P (% of total)
Maize	0.26	0.22	84.6
Brown rice (unpolished)	0.38	0.28	73.7
Rice (polished)	0.31	0.17	54.8
Wheat bran	1.15	1.03	76.9
Cassava	0.16	0.04	25
Sweet potato	0.21	0.05	23.8
Potato	0.24	0.05	20.8
Soy bean	0.6	0.37	61.7
Lentils	0.31	0.2	64.5

3. Health Impacts

It has been widely reported that disturbances in systemic phosphorus homeostasis leading to both high and low serum phosphorus levels have been associated with an increase in the risk of cardiovascular diseases. Hyperphosphatemia promote vascular calcification and endothelial dysfunction, with only a small elevation of serum levels within the normal range capable of provoking such effects, even in individuals free of cardiovascular and chronic kidney diseases (Gutiérrez, 2013; Tanaka et al., 2013; Uribarri and Calvo, 2013).

Furthermore, hypophosphatemia is also reportedly associated with insulin resistance and impaired glucose tolerance, while the addition of phosphorus in an

OGTT was found to improve insulin sensitivity and reduce blood glucose levels by increasing the rate of glucose disposal (Khattab et al., 2011).

Dietary phosphorus was also found to influence the development of fatty liver induced by high cholesterol intakes. Tanaka et al. (2013) showed that dietary phosphorus restriction in mice significantly decreased plasma cholesterol levels while increasing liver weights and hepatic lipid accumulation induced by a high cholesterol diet. Low phosphorus status might also be an underlying cause behind many other diseases such as hypertension and the development of obesity (Obeid, 2013).

The role of phosphorus in bone health has been extensively documented. Excessive dietary phosphorus intake and hyperphosphatemia negatively affect bone health by disrupting the hormonal regulation of phosphorus, calcium and vitamin D leading to a disordered mineral metabolism and bone loss (Calvo and Tucker, 2013; Takeda et al., 2014; Uribarri and Calvo, 2013). In contrast, Henry et al. (1979) stated that a severe phosphorus depletion induce a decrease in bone mineralization and protein deposition in tissues.

4. Phosphorus and Growth

Concerning the impact of phosphorus on growth, to this day not much research has been conducted. Henry et al. (1979) reported that phosphorus depletion to the quarter of normal levels in growing rats induced a decrease in growth rate, energy consumption and energy efficiency as well as protein efficiency and protein deposition in tissues. Moreover, the inclusion of phosphorus after a deficiency led to the improvement of growth. In another study (Pastoor et al., 1995) the restriction of phosphorus, to half the minimum amount required, slightly decreased weight gain, food intake and tibia growth in female kittens. But also, both plasma and urinary phosphorus

concentration were reduced as compensatory mechanisms to sustain normal growth. In addition, Tang et al. (2012) found that increasing dietary phosphorus in catfish improved growth by increasing body weight and feeding rate while body fat significantly decreased. Visanuvimola and Bertramb (2010) also showed that in crickets, the reduction of phosphorus availability in the diet induced a decrease in growth rates, while high phosphorus diets led to an increase in both weight gain and food intake.

More recently, in a study on rats (Hammoud et al., 2015), the addition of dietary phosphorus to a low complete protein diet (10% egg white protein) induced an increase in weight gain and food intake comparable to that observed with a normal protein diet (20% egg white protein).

Therefore, the objective of this study was to dissect the effect of phosphorus addition on weight gain, food intake, body composition, liver composition and other parameters in 40 rats maintained on a diet with wheat gluten as the exclusive source of protein.

CHAPTER III

MATERIAL AND METHODS

A. Experimental Procedure

This study received approval from the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB).

1. Animal Housing and Adaptation

Forty six-week old male Sprague-Dawley rats (American University of Beirut, Lebanon) weighting around 220g were used. They were housed in individual wire-bottomed cages that enable the collection of food spillage, in a temperature-controlled room (22°C ± 1°C) under 12-h/12-h light/dark cycle (lights on at 8 AM). Rats were allowed a seven days adaptation period where they had free access to water and were fed an ad libitum control diet (Table 6) to familiarize with the environment and the food before being put on their corresponding diets.

Table 6: Dietary composition of the control diet

Ingredients	Quantities (g/kg)
Caseine	198
Methionine	2
Corn starch	310
Sucrose	310
Corn oil	100
Cellulose	35
Mineral mix[1]	35
Vitamin mix[2]	10

[1] Mineral mix (AIN-93G, used at 35 g/kg of diet, obtained from Dyets inc.)
[2] Vitamin mix (AIN-93VX, used at 10 g/kg of diet, obtained from Dyets inc.)

2. Experimental Design and Diet

Following the one-week adaptation period, the rats were randomly divided into four experimental groups of ten rats, each group following its respective diet for a period of nine weeks (Table 7).

The experimental diets were isocaloric, all composed of wheat gluten as the only source of protein, with the percentage of energy from protein fixed at 10% for all four groups. The remaining dietary ingredients in common to all are corn starch, sucrose, corn oil, cellulose, a phosphorus free mineral mix and a vitamin mix. The groups only differ by the addition of lysine or phosphorus as follows:

- Group G: gluten diet, with no lysine and no phosphorus.
- Group G+L: inclusion of 0.6% lysine to the gluten diet.
- Group G+P: 0.3% phosphorus included in the form of potassium phosphate.
- Group G+L+P: presence of both 0.6% lysine and 0.3% phosphorus.

Table 7: Dietary composition of the experimental diets of the four groups

Ingredients g/kg	Group G	Group G+L	Group G+P	Group G+L+P
Wheat gluten [1]	130	130	130	130
Corn starch	345	342	338.4	335.4
Sucrose	345	342	338.4	335.4
Fat (corn oil)	100	100	100	100
Cellulose	35	35	35	35
Mineral mix [2]	35	35	35	35
Vitamin mix [3]	10	10	10	10
Phosphorus added [4]	-	-	13.2	13.2
Lysine added	-	6	-	6
Total calories (Kcal)	4180 Kcal (4.18 Kcal/g)	4180 Kcal (4.18 Kcal/g)	4127 Kcal (4.12 Kcal/g)	4127 Kcal (4.12 Kcal/g)

[1] The diets contains 10% protein coming exclusively from wheat gluten (which has 76% protein) [2] Phosphorus free mineral mix (AIN-93G mineral mix phosphorus free, used at 35 g/kg of diet, obtained from Dyets inc.) [3] Vitamin mix (AIN-93VX vitamin mix, used at 10 g/kg of diet, obtained from Dyets inc.) [4] The phosphorus used is potassium phosphate MW KH2PO4 =136 g of which P = 31g, in 1kg of diet for group 3 and 4: 13.2 g KH2PO4 are to be added to get 0.3% phosphorus

The rats were fed their corresponding experimental diets for nine weeks during which their body weight and food intake were measured twice per week, and the values for each week obtained by calculating the average of both days. Food intake calculation was done by subtracting the weight of the food cup on the day of weighting from its weight from the previous day of weighting, taking into account any possible food spillage.

After nine weeks of follow up, the rats were fasted overnight. The following morning they were placed under anesthesia and then dissected. Blood samples were drawn from each rat and collected in EDTA tubes that were directly placed on ice. The rats were sacrificed by severing their hearts. The livers were immediately excised and weighed; they were frozen in liquid nitrogen then stored at -80°C pending analysis and

fat extraction. Rat weights were recorded once after anesthetizia and another time after blood draw and liver excision. The rat carcasses were placed in a convection oven for drying. The blood samples were centrifuged at 3500 (g) for 15 minutes at 3°C and plasma aliquots were collected and stored at -80°C pending analysis.

B. Body Composition Analysis

The rat carcasses were dried in a convection oven for four days at 100°C. Weighing was done before and after drying to record wet and dry weights. The rats were then homogenized. Fat extraction was performed on samples of the carcasses in duplicate using petroleum ether solvent (BP 40-60°C) in the solvent recovery extractor for fats "Det-gras N" (J.P. Selecta, Barcelona, Spain). Fat weight of the samples was obtained, and total fat percentage was deduced.

C. Blood Analysis

1. Plasma Glucose, Lipid Profile, Phosphorus, Albumin, BUN and CRP

Plasma glucose, total cholesterol, HDL cholesterol, triglycerides, total phosphorus, albumin, BUN and CRP were determined using an enzymatic colorimetric method on the Vitros 350 Chemistry System (Ortho-Clinical Diagnostics, Johnson & Johnson, New York).

LDL cholesterol was established from the values of total cholesterol, HDL cholesterol and triglycerides using Friedwald equation:

LDL chol = Total chol – HDL chol – TG/5

2. Plasma Insulin

Plasma insulin concentration was determined by enzyme immunoassay using a Rat/Mouse Insulin Elisa Kit (EZRMI-13K) provided by EMD Millipore Corporation, USA.

D. Liver Analysis: Hepatic Fat Extraction

The liver samples were weighed before and after freeze-drying (2.5 Liter Bench top Freeze-Dry System, LABCONCO). After freeze-drying for 48 hours, the liver sections were crushed. Fat extraction was then performed on liver samples of around 1 g each, placed in moisture free sealable filter bags and weighed before and after extraction. Extraction was done for 40 minutes per run using petroleum ether solvent (BP 400-600C) in Ankom XT10 (USA). Fat weight was calculated by subtracting the weight difference of the samples before and after fat extraction; and fat percentage was deduced.

E. Statistical Analysis

Data are expressed as means ± SD of all values. Statistical analysis was performed using SPSS 22 software program. Results were analyzed by a two-way analysis of variance (ANOVA), with lysine and phosphorus as both variables, and the interaction representing lysine x phosphorus. A probability of less than 0.05 was considered to be significant.

CHAPTER IV

RESULTS

A. Food Intake and Body Weight

1. Food Intake

All values are expressed as mean ± SD. Food intake in grams per day, was recorded twice per week over a 9-week period, however the average of the two values for each week are presented for the first 8 weeks (Table 9) as well as the total food intake of the 9 weeks (Table 8). A two-way analysis of variance (ANOVA) was performed with lysine and phosphorus as the two variables.

The mean total food intake was the lowest in the group containing only gluten as the source of protein in group G (Table 8 and Figure 1). It was found that both lysine and phosphorus significantly affected food intake, since the inclusion of either lysine (group G+L) or phosphorus (group G+P) to the gluten diet increased total food intake by about 15%. However, the presence of their combination (lysine and phosphorus) in the group G+L+P was associated with about a 45% increment in food intake, which was more attributed to the added effect of lysine and phosphorus since their interaction was found close to significant (P=0.058) (Table 8).

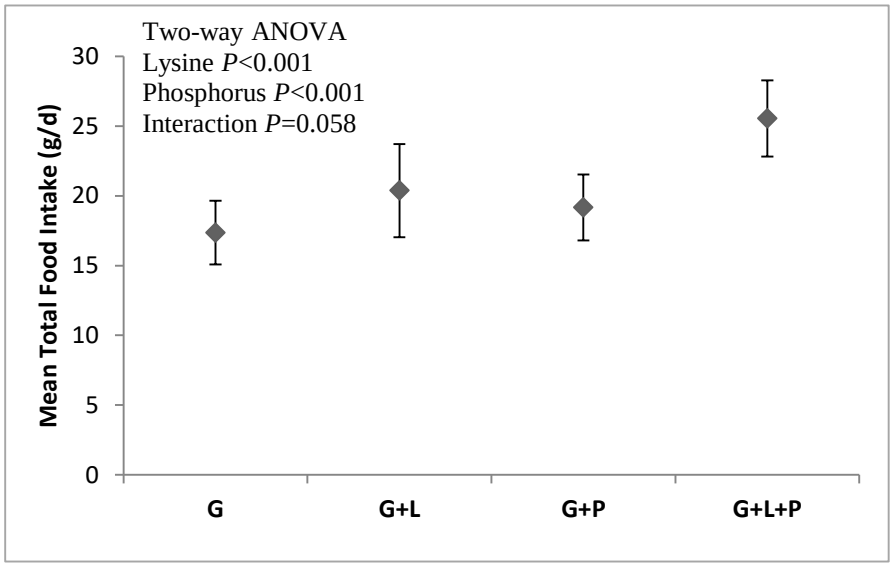
And this was observed through the first 8 weeks of the experiment (Table 9 and Figure 2), where food intake was affected by lysine and phosphorus, while the effect of their interaction was mostly not significant. As for food intake variation with time within each group, no statistical significance was found with groups G+L, G+P and G+L+P (P>0.05), while a slight decrease in the second half of the experiment was observed for group G, and food intake of G+L+P was at each week higher than the other groups.

Table 8: Body weight (g), weight gain (g/d), food intake (g/d) and energy efficiency (g/100Kcal) of the four groups of rats maintained on varied gluten diets over a 9-week experimental period

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Initial body weight (g)	227.26±17.45	225.88±18.58	227.28±18.17	226.94±17.43	0.880	0.925	0.927
Final body weight (g)	236.87±22.38	289.72±36.27	281.39±23.65	473.57±60.39	<0.001	<0.001	<0.001
Total food intake (g/d)	17.36±2.28	20.37±3.34	19.17±2.37	25.54±2.73	<0.001	<0.001	0.058
Total weight gain (g/d)	0.152±0.245	1.013±0.382	0.859±0.314	3.915±0.905	<0.001	<0.001	<0.001
Total EE (g/100Kcal)	0.213±0.335	1.199±0.432	1.064±0.351	3.627±0.478	<0.001	<0.001	<0.001

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

Figure 1: Mean total food intake in grams per day of the four groups over the 9-week experimental period



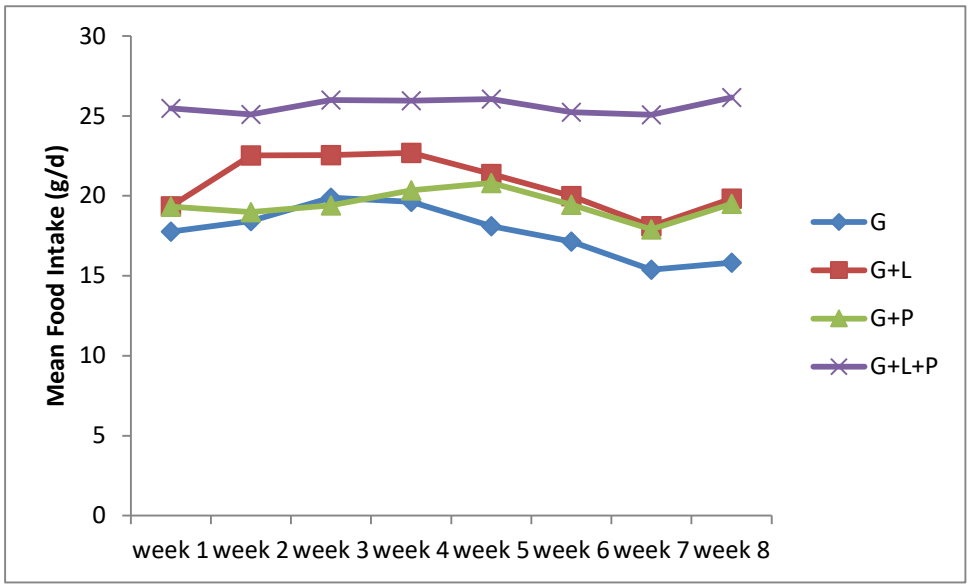
Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

Table 9: Average food intake in grams per day of the four groups presented weekly, during the first 8 weeks of the experimental period

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Week 1	17.770±1.331^{abcd}	19.344±2.513	19.327±2.619	25.47±4.13	<0.001	<0.001	0.015
Week 2	18.434±2.465^{abc}	22.53±5.31	18.993±2.974	25.10±3.61	<0.001	0.195	0.401
Week 3	19.88±4.28^{a}	22.54±4.34	19.40±3.48	25.997±2.830	<0.001	0.221	0.109
Week 4	19.62±4.02^{ab}	22.69±4.40	20.347±3.031	25.96±4.04	<0.001	0.114	0.309
Week 5	18.109±2.537^{abc}	21.37±3.66	20.811±2.209	26.05±3.32	<0.001	<0.001	0.304
Week 6	17.136±2.322^{bcd}	19.98±4.93	19.454±2.232	25.239±2.441	<0.001	0.001	0.154
Week 7	15.376±2.227^{d}	18.11±4.64	17.914±1.978	25.073±2.553	<0.001	<0.001	0.027
Week 8	15.82±3.87^{cd}	19.82±4.01	19.500±2.825	26.15±3.66	<0.001	<0.001	0.255
OWA *P*	0.012	0.145	0.425	0.988			

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed between the four groups, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. One-way ANOVA (OWA) was done to compare the within group variation with time. Significance is found with *P*-value < 0.05, different subscripts indicate significant difference using Fisher's test.

Figure 2: Mean food intake in grams per day presented weekly of the four groups over the first 8 weeks of the experimental period



Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

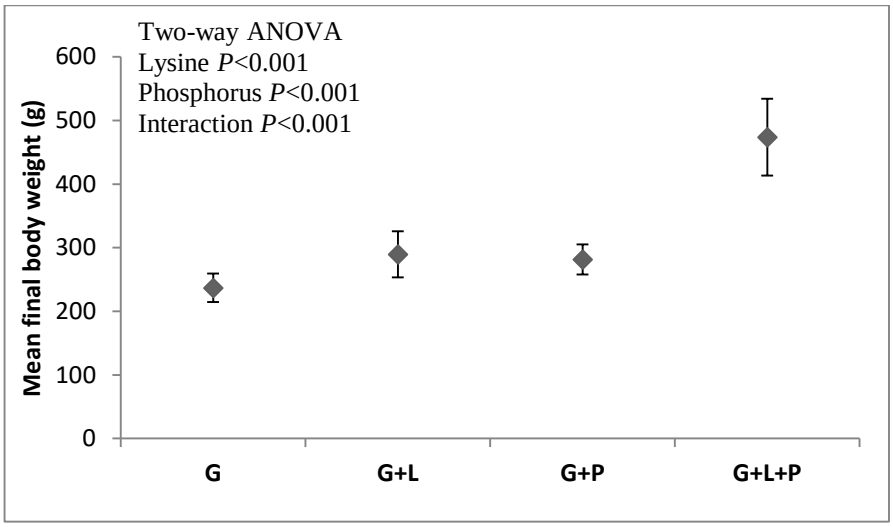
2. Body Weight and Weight Gain

The mean initial body weight of the rats was similar in between the four groups. Mean final body weight was recorded at the end of week 9 before the rats were sacrificed. Body weight was measured twice per week during 9 weeks, data for weight gain expressed as grams gained per day was averaged for every week presented for the first 8 weeks (Table 10), and the total weight gain was also calculated (Table 8).

Lysine, phosphorus and the interaction of both exhibited significant effects on mean final body weight and mean total weight gain. In the gluten diet lacking lysine and phosphorus (group G), weight gain was blunted with mean final body weight (Figure 3) and mean total weight gain (Figure 4) having the lowest values of all four groups. Mean total weight gain increased by about 5 times with the presence of either lysine (group G+L) or phosphorus (group G+P) in the diet, while the addition of lysine and phosphorus together (group G+L+P) amplified this effect by increasing total weight gain by about 20 folds compared to group G (Figure 4).

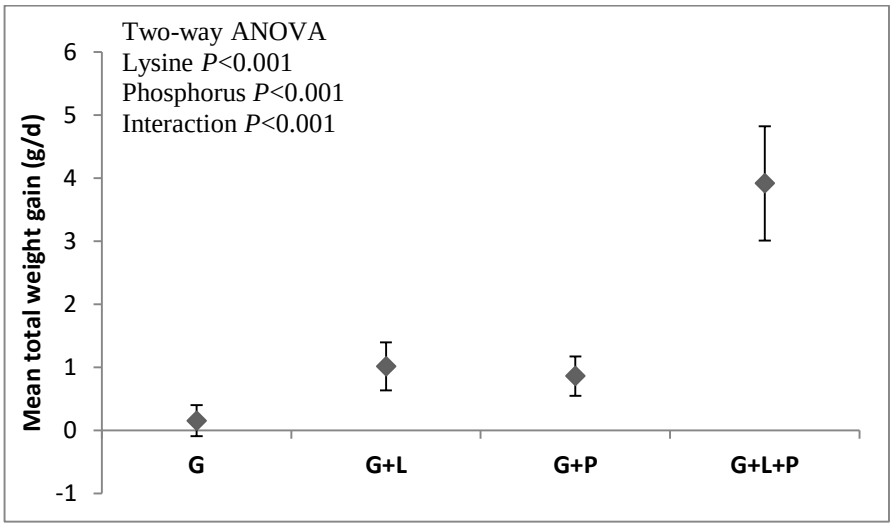
This trend was observed during 8 weeks since the effect of all of lysine, phosphorus and the interaction on weight gain was significant at each week, and weekly weight gain in G+L+P was at each time point higher than that of the other groups (Table 10 and Figure 5).

Figure 3: Mean final body weight in grams of the four groups over the 9-week experimental period



Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

Figure 4: Mean total weight gain in grams per day of the four groups over the 9-week experimental period



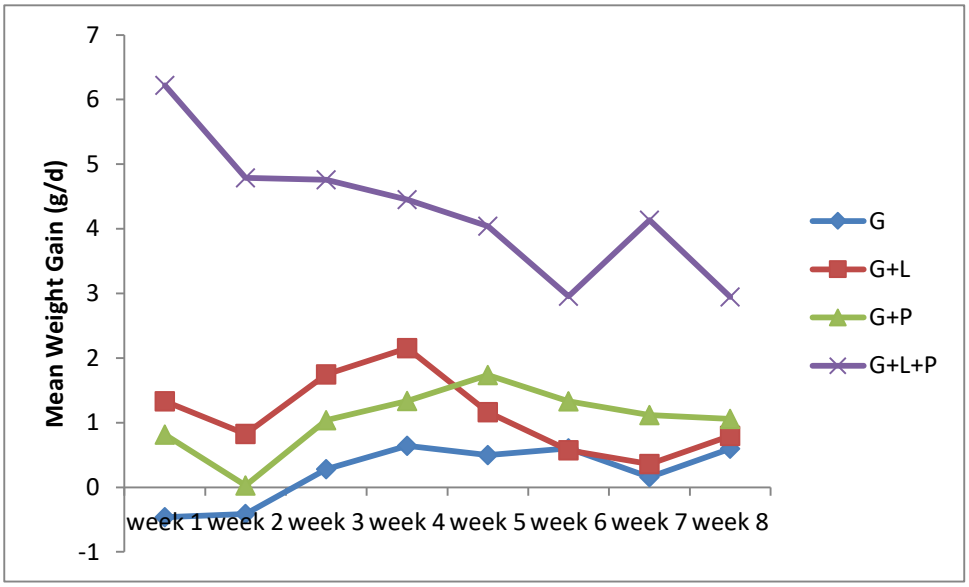
Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

Table 10: Average weight gain in grams per day of the four groups presented weekly, during the first 8 weeks of the experimental period

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Week 1	-0.461±0.566^{a}	1.330±0.912^{abc}	0.817±1.059^{a}	6.219±2.57^{a}	<0.001	<0.001	<0.001
Week 2	-0.414±0.488^{a}	0.824±1.166^{bc}	0.029±0.942^{b}	4.787±1.755^{ab}	<0.001	<0.001	<0.001
Week 3	0.281±0.504^{b}	1.749±0.627^{ab}	1.040±0.728^{ac}	4.760±1.581^{b}	<0.001	<0.001	0.001
Week 4	0.641±0.344^{b}	2.154±0.742^{a}	1.337±1.079^{ac}	4.450±1.803^{b}	<0.001	<0.001	0.031
Week 5	0.500±0.282^{b}	1.160±1.359^{abc}	1.737±0.614^{c}	4.041±1.157^{bc}	<0.001	<0.001	0.010
Week 6	0.603±0.753^{b}	0.573±0.837^{c}	1.329±0.720^{ac}	2.953±0.915^{c}	0.004	<0.001	0.003
Week 7	0.157±0.612^{b}	0.361±1.945^{c}	1.120±0.748^{ac}	4.134±0.935^{bc}	<0.001	<0.001	0.001
Week 8	0.596±0.887^{b}	0.796±1.771^{bc}	1.059±0.510^{ac}	2.944±1.584^{c}	0.015	0.003	0.047
OWA *P*	<0.001	0.033	0.002	0.001			

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed between the four groups, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. One-way ANOVA (OWA) was done to compare the within group variation with time. Significance is found with *P*-value < 0.05, different subscripts indicate significant difference using Fisher's test.

Figure 5: Mean weight gain in grams per day presented weekly of the four groups over the first 8 weeks of the experimental period



Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

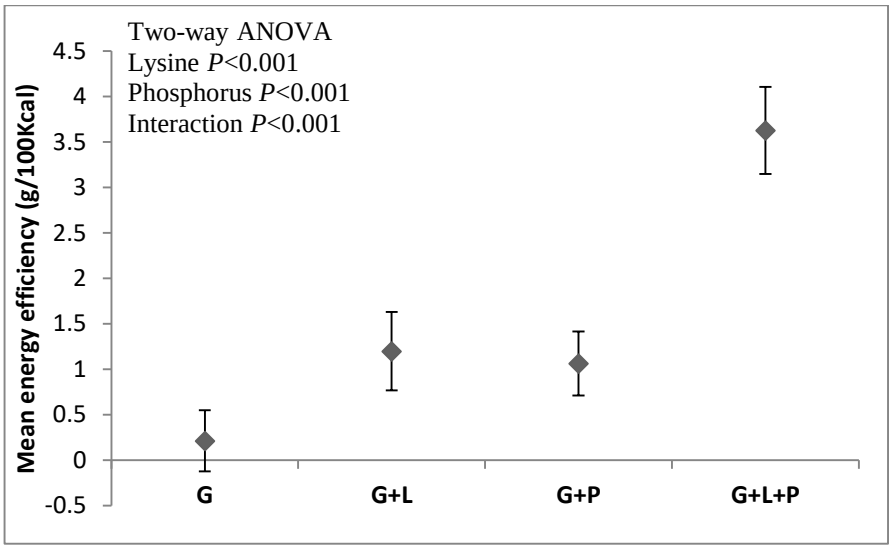
3. Energy Efficiency

Energy efficiency was calculated as weight gained for 100 Kcal of food consumed and the average for each week during the first 8 weeks (Table 11) as well as the total energy efficiency (Table 8) are presented.

The variation of energy efficiency is similar to that of weight gain with lysine, phosphorus and their interaction all found to have a significant effect. The lowest value was observed in group G (Figure 6), but energy efficiency increased with the addition of lysine (group G+L) or phosphorus (group G+P) to the gluten diet, with the magnitude of the increase (5 times) similar between the two groups. However, the interaction between lysine and phosphorus (group G+L+P) increased energy efficiency by about 20 folds.

This was also true during 8 weeks, since the weekly energy efficiency was always greater in group G+L+P as compared to the others (Table 11 and Figure 7). However, phosphorus alone maintained a significant effect on energy efficiency at each time point, while the effect of lysine and the interaction was at some weeks found insignificant ($P>0.05$).

Figure 6: Mean total energy efficiency in grams per 100 Kcal of the four groups over the 9-week experimental period



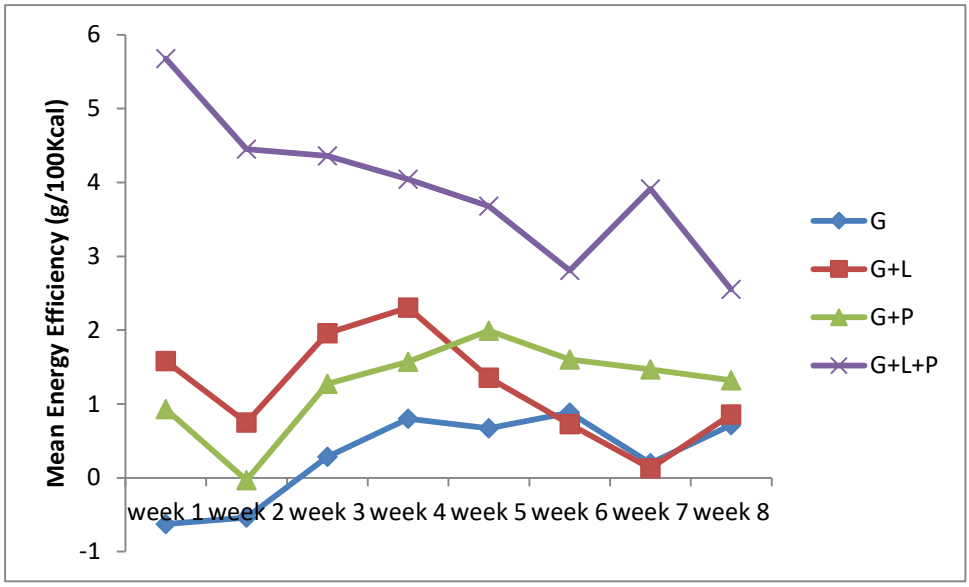
Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

Table 11: Average energy efficiency in grams per 100 Kcal of the four groups presented weekly, during the first 8 weeks of the experimental period

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Week 1	-0.624±0.791[a]	1.580±0.996	0.931±1.215[a]	5.676±1.260[a]	<0.001	<0.001	0.001
Week 2	-0.540±0.594[ab]	0.749±1.443	-0.033±1.288[b]	4.449±1.036[b]	<0.001	<0.001	<0.001
Week 3	0.284±0.667[c]	1.960±0.845	1.273±0.886[ac]	4.358±1.215[b]	<0.001	<0.001	0.021
Week 4	0.800±0.456[c]	2.304±0.721	1.573±1.217[ac]	4.044±1.144[b]	<0.001	<0.001	0.127
Week 5	0.672±0.402[c]	1.357±1.572	1.991±0.666[c]	3.681±0.788[bc]	<0.001	<0.001	0.107
Week 6	0.882±1.134[c]	0.728±1.130	1.602±0.797[ac]	2.806±0.809[cd]	0.099	<0.001	0.035
Week 7	0.197±1.088[bc]	0.129±2.748	1.470±0.915[ac]	3.914±0.589[b]	0.022	<0.001	0.016
Week 8	0.713±1.441[c]	0.859±2.275	1.322±0.661[ac]	2.554±1.276[d]	0.162	0.022	0.268
OWA *P*	<0.001	0.065	0.001	<0.001			

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed between the four groups, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. One-way ANOVA (OWA) was done to compare the within group variation with time. Significance is found with *P*-value < 0.05, different subscripts indicate significant difference using Fisher's test.

Figure 7: Mean energy efficiency in grams per 100 Kcal presented weekly of the four groups over the first 8 weeks of the experimental period



Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

B. Body Composition Analysis

Water, fat and defatted weight (g) in the whole carcass of the rats were significantly higher (P<0.001) with the addition of lysine (G+L) or phosphorus (G+P) to the gluten diet as compared to group G (Table 12). But the highest values were observed in group G+L+P resulting from the interaction of lysine and phosphorus.

In term of percentages (Table 13), % of dry weight and fat % in the whole carcass were found to increase with the addition of phosphorus (G+P), but even more with the presence of both lysine and phosphorus (G+L+P). As for water % and defatted %, both decreased with the addition of phosphorus (G+P), and a bigger reduction was witnessed with the presence of both lysine and phosphorus (G+L+P).

Lastly, considering the mineral mass as stable and negligible, protein is represented by the defatted mass remaining after subtracting the water and fat weight from the carcass. Phosphorus alone held a significant effect on the defatted % (P<0.001), which was found higher in the groups G+P and G+L+P as compared to those bared of phosphorus (G and G+L) (Table 13).

Table 12: Mean water, fat and defatted weight in grams of the four rat groups

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Wet weight (g)	231.73±21.47	281.9±34.5	274.46±23.70	463.6±59.3	<0.001	<0.001	<0.001
Water weight (g)	157.44±14.54	191.47±23.19	178.79±17.18	254.55±18.72	<0.001	<0.001	<0.001
Dry weight (g)	74.29±7.74	90.45±11.58	95.67±8.11	209.0±61.2	<0.001	<0.001	<0.001
Fat weight (g)	14.232±1.335	17.65±3.30	21.21±3.25	78.0±38.6	<0.001	<0.001	<0.001
Defatted from dry (g)	60.06±6.71	72.80±9.20	74.46±6.69	130.98±26.50	<0.001	<0.001	<0.001

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with P-value < 0.05.

Table 13: Mean percentage of water, fat, defatted mass and protein in the four rat groups

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Water %	67.949±1.166	67.930±0.743	65.109±1.493	55.60±6.75	<0.001	<0.001	<0.001
Fat in wet %	6.157±0.432	6.239±0.786	7.762±1.220	16.29±5.78	<0.001	<0.001	<0.001
Defatted %	25.894±1.023	25.831±0.943	27.129±0.790	28.108±2.606	0.370	0.001	0.296
Dry weight %	32.051±1.166	32.070±0.743	34.891±1.493	44.40±6.75	<0.001	<0.001	<0.001
Fat in dry %	19.210±1.178	19.451±2.406	22.165±2.755	35.92±7.82	<0.001	<0.001	<0.001
Defatted from dry %	80.790±1.178	80.549±2.406	77.835±2.755	64.08±7.82	<0.001	<0.001	<0.001

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with P-value < 0.05.

C. Liver Composition Analysis

Liver wet and dry weight (g) both increased with the addition of lysine (G+L) and phosphorus (G+P) as compared to group G, but the highest increment was observed in group G+L+P where the weight about doubled following the combination of lysine and phosphorus in the diet (Table 14). However, when taking into consideration the total body weight, liver weight (g/100g BW) was not influenced by the interaction of lysine and phosphorus (P=0.995) but increased in groups G+L and G+P, and even more in the group G+L+P because of the addition of each of lysine and phosphorus independently of the other.

Regarding the percentages of liver dry weight, water, fat and defatted, they were only significantly affected by the addition on phosphorus (Table 15). The % of liver dry weight increased in the groups G+P and G+L+P, and in contrast water % decreased in the same groups. Groups G+P and G+L+P also had higher % of fat as compared to groups G+L and G. As for defatted %, that represents the protein as mineral mass is considered stable, it was the lowest in group G+P.

Table 14: Liver water, fat and protein weight of the four rat groups

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Liver wet weight (g)	6.500±0.657	8.589±1.338	8.446±1.566	15.108±2.946	<0.001	<0.001	<0.001
Liver wt g/100g BW	2.746±0.150	2.965±0.293	2.983±0.335	3.175±0.376	0.039	0.026	0.995
Liver water weight (g)	4.676±0.459	6.144±0.956	5.827±1.007	10.465±2.074	<0.001	<0.001	<0.001
Liver dry weight (g)	1.824±0.213	2.445±0.405	2.619±0.641	4.643±1.075	<0.001	<0.001	0.003
Liver fat weight (g)	0.199±0.061	0.289±0.096	0.703±0.460	0.960±0.384	0.078	<0.001	0.332

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with P-value < 0.05.
(wt: weight, BW: body weight)

Table 15: Liver water, fat and protein percentages of the four rat groups

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Liver water %	71.958±1.033	71.540±1.254	69.181±2.939	69.31±3.44	0.851	0.002	0.721
Liver fat in wet %	3.046±0.826	3.363±0.895	8.03±4.25	6.365±2.332	0.487	<0.001	0.292
Liver defatted %	24.997±1.055	25.096±0.923	22.787±1.574	24.324±1.961	0.081	0.002	0.123
Liver dry weight %	28.042±1.033	28.460±1.254	30.819±2.939	30.69±3.44	0.788	0.003	0.717
Liver fat in dry %	10.839±2.883	11.757±2.768	25.15±11.07	20.36±5.67	0.440	<0.001	0.250

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with P-value < 0.05.

D. Plasma Analysis

1. Phosphorus, Albumin, BUN and CRP

Mean plasma phosphorus concentration was significantly affected by the presence of phosphorus and was higher in the groups G+P and G+L+P (Table 16).

Lysine as well as the interaction lysine-phosphorus had a significant effect on mean plasma albumin concentration. The lowest value was observed in the group G+P, followed by that of group G, while the addition of lysine increased plasma albumin (group G+L), with the highest concentration attributed group G+L+P with the presence of lysine and phosphorus as shown in Table 16.

Mean BUN concentration significantly decreased with the addition of phosphorus to the diet (group G+P and group G+L+P), but was not affected by lysine or the interaction lysine-phosphorus (Table 16).

There was no statistically significant effect on plasma CRP levels (Table 16).

Table 16: Mean plasma total phosphorus, albumin, BUN and CRP of the four groups

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Total P (mg/dl)	5.989±1.359	5.790±1.116	7.490±0.782	8.260±0.643	0.425	<0.001	0.167
Albumin (g/dl)	3.133±0.158	3.410±0.213	2.770±0.216	3.744±0.321	<0.001	0.850	<0.001
BUN (mg/dl)	15.78±7.97	16.00±7.01	9.30±5.77	11.70±2.98	0.511	0.010	0.585
CRP (mg/L)	0.889±1.364	0.600±0.699	1.300±1.567	0.700±0.483	0.218	0.519	0.642

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.
(P: phosphorus, BUN: Blood Urea Nitrogen, CRP: C-reactive protein)

2. Glucose, Insulin and HOMA-IR

There was no significant effect on mean plasma glucose concentration by either lysine, phosphorus or both (Table 17).

Lysine, phosphorus and the interaction lysine-phosphorus significantly affected mean plasma insulin levels, which slightly increased in groups G+L and G+P as compared to group G, while a higher increase was seen in group G+L+P (Table 17).

Concerning HOMA-IR index (Table 17), it was significantly affected by each of lysine and phosphorus, but not by their interaction. A very small increase from group G was detected with the addition of lysine (group G+L) or phosphorus (group G+P), but a higher increment was induced by the presence of both (group G+L+P).

Table 17: Mean plasma glucose, insulin and HOMA-IR index of the four groups

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Glucose (mg/dl)	177.0±60.3	184.7±63.2	171.0±56.4	233.5±42.2	0.052	0.215	0.121
Insulin (μg/ml)	491.0±58.7	516.0±52.0	521.2±177.4	703.4±117.4	0.007	0.005	0.032
HOMA-IR	6.345±2.610	6.906±2.832	6.86±5.29	11.596±2.496	0.025	0.027	0.073

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with P-value < 0.05.

3. Lipid Profile

Phosphorus alone, but not lysine or the interaction of lysine and phosphorus, had a significant effect on cholesterol (Table 18). Mean plasma concentration of total cholesterol, LDL cholesterol and HDL cholesterol all decreased in the two groups with added phosphorus (group G+P and group G+L+P).

Mean plasma levels of triglycerides increased with the addition of lysine alone (group G+L) as compared to the groups with no lysine (group G and group G+P), but was also affected by the interaction of lysine and phosphorus with an even higher increment observed with the addition of both to the diet (group G+L+P) in table 18.

Table 18: Mean plasma triglycerides, total and LDL and HDL cholesterol of the four groups

					Two-way ANOVA *P*-value		
	G	G+L	G+P	G+L+P	Lysine	Phosphorus	Interaction
Cholesterol (mg/dl)	89.22±13.80	84.70±13.27	75.20±10.13	81.20±6.78	0.982	0.014	0.216
LDL (mg/dl)	16.53±4.32	17.50±5.29	12.32±5.41	12.32±4.37	0.799	0.006	0.732
HDL (mg/dl)	67.33±10.30	59.50±9.57	58.10±6.31	57.20±4.92	0.074	0.023	0.252
TG (mg/dl)	26.78±6.48	38.50±9.55	23.90±5.43	58.40±26.43	<0.001	0.117	0.034

Group G contains 10% gluten protein; Group G+L: inclusion of lysine in the gluten diet; Group G+P: inclusion of phosphorus in the gluten diet; Group G+L+P: inclusion of lysine and phosphorus in the gluten diet. Values for each group are expressed as mean ± SD (standard deviation). Two-way ANOVA was performed, with lysine and phosphorus as the two variables, and the interaction representing lysine x phosphorus. Significance is found with *P*-value < 0.05.

CHAPTER V

DISCUSSION

Wheat proteins, mostly in the form of gluten, are plant proteins of low quality, lacking primarily in the essential amino acid lysine (Bos et al., 2005). Many developing countries, as well as individuals following vegetarian or vegan diets, highly depend on wheat as the major source of both energy and protein (Hussain et al., 2004; Zhao et al., 2004). Wheat contains a moderate quantity of gluten proteins (around 10%), and when used as the main dietary protein source, those incomplete proteins cannot sustain optimal growth and were reported to induce growth impairment (Burns et al., 1982). Wheat is also known to contain limited amount of available phosphorus, which has recently been found to improve growth in a low complete protein diet (Hammoud et al., 2015). Therefore, this study used an animal model (rats) to test the hypothesis that phosphorus can impact growth maintained on an incomplete protein diet.

The variation of protein levels in the diet have been reported to strongly influence food intake. In previous studies, a U shaped relation between both was established. Very low protein diets as well as high dietary protein led to a decrease in food consumption, while in contrast a moderately low protein diet (10% protein) induced an adaptative mechanism and was found to boost food intake (Du et al., 2000; Malta et al., 2014; Peng et al., 1974; White et al., 2000). In the case of our experiment, no down regulation of food intake was observed, as the within group variation with time was not significant in the groups G+L, G+P and G+L+P. Moreover, changes in food intake were apparent from the start of the experimental period, which indicates an acute

effect. Radcliffe and Webster (1976) stated that animals are able to control their appetite to serve two goals: long term control where the needs for growth determine nutrient requirements and short term regulation of food intake to maintain homeostasis. It was in fact reported that lysine deficient rats are extremely sensitive to small reductions in dietary lysine concentration. These rats would, when given the choice, select against such diets in order to meet the needs for growth (Hrupka et al., 1999) and would quickly adapt food intake to avoid consuming an amino acid imbalanced diet (Leung et al., 1968).

The significantly lower food intake seen in group G as compared to the other groups is evidently due to the low quality protein of the diet and its inadequacy in term of lysine, which is in line with previous studies reporting that lower amount of food was consumed when the proteins in the diet were of low quality (Burns et al., 1982; Mattar and Obeid, 2010). A possible explanation for this manifestation is related to the decline of plasma lysine levels following the consumption of a diet limited in this amino acid, which would result in lower concentration of lysine in the brain inducing an anorectic response to decrease food intake (Gietzen et al., 1998; Harper et al., 1970). Another possibility may be that the limited amount of a certain essential amino acid in an incomplete protein diet will lead to an excess in other amino acid, notably because of the incapacity to optimally metabolize proteins. A decrease in food intake would follow in order to protect against the potential toxicity of this amino acid accumulation (Du et al., 2000).

The addition of lysine to the gluten diet (group G+L), as well as that of phosphorus in group G+P, were each found to increase food intake by about 15%. These results are supported by other studies where energy consumption was enhanced

with the increase of dietary lysine (Chang and Chao, 1969; Mattar and Obeid, 2010) or the addition to the diet of phosphorus (Hammoud et al., 2015). However, supplementing with lysine alone was not always able to highly improve weight gain and food intake (Hussain et al., 2004; Zhao et al., 2004), and better results were observed when lysine was added in combination with threonine (Tanphaichitr et al., 1976). On the other hand, ATP production is known to be directly associated with phosphorus availability, and an adequate supply of dietary phosphorus is required for postprandial hepatic ATP production (Obeid et al., 2014), hence making ATP available for protein synthesis. A sufficient amount of phosphorus would thereby facilitate amino acid metabolism and raise energy intake (Hammoud et al., 2015; Henry et al., 1979), that has been found to decrease with the accumulation of free amino acid in the circulation (Young and Marchini, 1990). Additionally, adding lysine plus phosphorus together in group G+L+P induced an increment in food intake of around 45%, and this seems to be due to the added effect of both since their combination led to a borderline significant increase (P = 0.058). These results suggest that the variation of food intake may not be related to the amount of dietary protein but rather to the capacity of the body to properly metabolize the ingested proteins.

The previous suggestion can be a possible explanation to the variation of body proteins and plasma BUN. Phosphorus, independently of lysine, induced a significant increase in the total protein content of the carcasses, as well as a decline in BUN plasma levels, a product of protein breakdown (Harper et al., 1970). The presence of phosphorus has thus improved protein metabolism and diminished amino acid oxidation and deamination. This is in accordance with the finding of Henry et al. (1979) that showed a decline in protein deposition in tissues occurring after severe phosphorus depletion, as it is well known that protein metabolism is highly depended on

phosphorylation. Therefore, the presence of an adequate amount of phosphorus, along with lysine, is necessary to get an optimal protein metabolism with incomplete protein diets.

Mean final body weight and weight gain were blunted in group G, as expected with incomplete protein diets in which an uneven supply of one of the indispensable amino acids can impair growth (Burns et al., 1982; Herzeberg and Rogerson, 1984; Mattar and Obeid, 2010). Very low protein diets are also well known to induce growth retardation (Donald et al., 1981; Swick et al., 1983). Even though the diet consumed by the rats in group G contains a moderately low amount of protein (10%), growth was impaired similarly to that of very low protein diets because of the imbalanced essential amino acid pattern of wheat gluten constituting the sole protein source in the diet. It was in fact proven that the dietary inadequacy of one of the essential amino acids will induce effects witnessed with protein deficiency namely growth retardation, body weight loss, diminished protein synthesis and amino acid oxidation (Ousterhout, 1960; Sidransky and Baba, 1960; Yamashita and Ashida, 1969).

The pattern of changes in weight gain was however different from that observed with food intake. Adding either lysine (group G+L) or phosphorus (group G+P) to the gluten diet lead to an increase in weight gain of about 5 times. The improvement in weight gain and growth observed with the addition to the gluten diet of lysine, the main limiting amino acid, were widely reported in past studies (Graham et al., 1981; Munaver and Harper, 1959; Yamashita and Ashida, 1969). Notably, Bahl and Venkitasubramanian (1977) found that the fortification of wheat by as little as 0.2% lysine induced significant improvement in weight gain of rats. However, our main objective was to decipher the role of phosphorus in such diets. Similarly to lysine,

adding phosphorus enhanced weight gain and growth, which was supported by the findings of Hammoud et al. (2015) where weight increased with the rise of the amount of dietary phosphorus. In this same context, Henry et al. (1979) showed that phosphorus addition after a deficiency improved growth rate. However, the addition of both lysine and phosphorus together in the diet (group G+L+P) amplified this effect, as the combination of those two variables induced a considerable higher increment in weight of around 20 folds. In our experiment, this large increase in weight gain observed in group G+L+P was not attributed to a greater consumption of food since the magnitude of increase observed in food intake was lower. Additionally, the diets administered to the four groups had similar compositions, and varied majorly by the addition of lysine or phosphorus. It has been reported that the rise in weight gain is not necessarily caused by hyperphagia (Racliffe and Webster, 1976), and in our case this was further explained when energy efficiency (weight gain in grams for 100 Kcal consumed) was calculated. A similar pattern of variation for mean weight gain and energy efficiency can be seen between the groups. Indeed, energy efficiency significantly improved with the addition of either lysine or phosphorus, and increased by about 5 times. This enhancement was intensified to a 20-fold increment with the combination of lysine and phosphorus (group G+L+P). Therefore, we can state that the improvement in weight gain and growth following the addition of lysine and phosphorus seems to be mainly related to a more efficient utilization of the energy consumed as seen with an enhanced energy efficiency, rather than an increased food intake. Adding each of lysine and phosphorus alone to the gluten diet induced only a small enhancement in weight and food intake. Since both lysine, as an essential amino acid, and phosphorus, being involved in ATP production, are required in good quantities to obtain an optimal metabolism, adding their combination is required to obtain the highest improvement in growth rate.

Lysine and its interaction with phosphorus were found to induce an increase in plasma albumin levels. This outcome was witnessed in other studies where lysine supplementation also enhanced serum albumin (Scrimshaw et al., 1973). This was even observed in infants who followed lysine deficient diets and had low serum albumin levels (Graham et al., 1981). It is well known that the imbalance in one essential amino acid will limit the utilization of other amino acids and the synthesis of body protein in the same way as protein deficiency (Ousterhout, 1960; Sidransky and Baba, 1960; Yamashita and Ashida, 1969). Additionally, it was reported that lysine is a major amino acid component of albumin (Denko et al., 1970) therefore its presence is vital for albumin synthesis. Phosphorus also plays a crucial role in protein metabolism as stated previously. All this can explain the rise in albumin plasma levels resulting from lysine addition and its interaction with phosphorus, indicating an increased protein synthesis.

Body fat was found to be significantly affected by lysine, phosphorus and lysine x phopshorus, however only phosphorus held a significant impact on liver fat. The fat in the whole rat carcass increases with the presence of phosphorus and a higher increment was observed with the combination of lysine plus phosphorus. As for liver fat, it was considerably higher in the groups containing phosphorus regardless of the presence of lysine. Dietary phosphorus has been found to influence the development of fatty liver following high cholesterol diets. Tanaka et al. (2013) showed that dietary phosphorus restriction in mice significantly decreased plasma cholesterol levels while increasing hepatic lipid accumulation. The opposite happened in our experiment where phosphorus addition, and not restriction, provoked such effects. Total cholesterol, LDL cholesterol and HDL cholesterol were affected by phosphorus and all significantly decreased in the two phosphorus containing groups. These findings are in line with those of Hazim et al. (2014) where phosphorus supplementation induced a decrease in

apoB100 indicating a possible suppression in hepatic production or increase in the clearance from the circulation of VLDL and LDL, the apoB100 containing lipoproteins. Moreover, the relation between plasma phosphorus and the lipid profile seems to be controversial as high serum phosphate was linked with both an increase and decrease in total and LDL cholesterol, however an inverse relation with triglycerides was observed in both studies (Lippi et al., 2009; Park et al., 2009).

In the case of our study, mean plasma triglyceride levels were found to increase with the addition of lysine alone, but were also affected by the interaction of lysine and phosphorus where an even higher increment was observed (G+L+P). The importance of lysine in the hepatic synthesis of carnitine has been widely documented. Carnitine is involved in the intra-mitochondrial transport of long-chain fatty acids groups for beta-oxidation (Tanphaichitr et al., 1976); and it was reported (Khan and Bamji, 1979) that dietary lysine deficiency would reduce carnitine levels, therefore impair fatty acid oxidation and result in lipid, notably triglyceride, accumulation in the body tissues. Tanphaichitr et al., 1976 went further stating that an essential amino acid deficiency, for instance lysine, would impair protein synthesis particularly that of the lipoprotein complex which is needed for hepatic triglyceride secretion, leading to an increased fat accumulation in the liver. Therefore, lysine will play a role in the formation of the lipoprotein complex as well as that of carnitine, thus an adequate amount of lysine is needed to prevent triglyceride accumulation in tissues. Moreover, plasma insulin was significantly higher in group G+L+P indicating a state of decreased insulin sensitivity. Insulin, a lipogenic hormone promoting fat storage, was widely reported to stimulate triglyceride synthesis, and insulin resistance has been associated with an impaired lipid profile and increased serum triglycerides (D'agostino et al., 2004; Dimitriadis et al.,

2011; Garg, 1996; McMurray and Hackney, 2005), further explaining the higher increment in triglycerides observed in group G+L+P.

It is good to note as a notable strength that, this study being done on an animal model, it was conducted in a well controlled environment, particularly regarding weight and food intake measurements.

CHAPTER VI

CONCLUSION

The addition of phosphorus to a diet based exclusively on wheat gluten, a low quality protein, exhibited similar increases in weight gain and food intake as the supplementation with lysine, the main limiting amino acid. However, the presence of both lysine and phosphorus had a greater impact and was able to further improve growth, which seemed to be mainly related to more efficient energy utilization rather than increased energy intake. Thus, a combination of the main missing amino acid with phosphorus is required to sustain optimal growth and enhance the quality of an incomplete protein diet.

Therefore, supplementing wheat derived products with a combination of phosphorus and lysine can be the best alternative in populations following plant based diets, especially in cases where complementing with a better quality protein, such as animal proteins, is expensive and thus not very affordable and feasible. For future researches, it would be interesting to conduct trials at a human level to observe the impact of this supplementation in humans.

BIBLIOGRAPHY

Amanzadeh J., & Reilly R.F. (2006). Hypophosphatemia: an evidence-based approach to its clinical consequences and management. *Nat. Clin. Pract. Nephrol., 2*(3): 136-148.

American Dietetic Association (ADA) (2003). Position of the American Dietetic Association and Dietitians of Canada: vegetarian diets. *J. Am. Diet. Assoc.,* 103(6): 748–765.

Anderson J.J.B. (2013). Potential health concerns of dietary phosphorus: cancer, obesity, and hypertension. *Ann. N.Y. Acad. Sci.,* 1301: 1–8.

Bahl S. and Venkitasubramanian T.A. (1977). Mechanism of Lipid Accumulation in Rats Fed Wheat Diets. *J. Nutr*., 107(8): 1385-1393.

Bazzano L.A., He J., Ogden L.G., Loria C.M., Vupputuri S., Myers L., Whelton P.K. (2002). Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. *Am. J. Clin. Nut.,* 76(1): 93–99.

Bos C., Juillet B., Fouillet H., Turlan L., Daré S., Luengo C., N'tounda R., Benamouzig R., Gausserès N., Tomé D., and Gaudichon C. (2005). Postprandial metabolic utilization of wheat protein in humans. *Am. J. Clin. Nut.,* 81(1): 87-94.

Burns R.A. LeFaivre M.H. Milner J.A. (1982). Effects of Dietary Protein Quantity and Quality on the Growth of Dogs and Rats. *J. Nut.,* 112(10): 1843-1853.

Calvo M.S. and Tucker K.L. (2013). Is phosphorus intake that exceeds dietary requirements a risk factor in bone health? *Ann. N. Y. Acad. Sci.,* 1301: 29–35.

Calvo M.S. and Uribarri J. (2013). Contributions to Total Phosphorus Intake: All Sources Considered. *Dialysis,* 26(1): 54–61.

Campbell W.W., Barton Jr. M.L., Cyr-Campbell D., Davey S.L., Beard J.L., Parise G., Evans W.J. (1999). Effects of an omnivorous diet compared with a lactoovovegetarian diet on resistance-training-induced changes in body composition and skeletal muscle in older men. *Am. J. Clin. Nutr.,* 70(6): 1032-1039.

Chang Y.O. and Chao N. (1969). Influence of various levels of lysine intake on weight gain and body composition in rats. *J. Agric. Food Chem.,* 17(1): 48-50.

Chase H.P., Welch N.N., Ganong J.L., Jansen G.R. (1976). Effects of Lysine and Threonine Fortification of Bread During Gestation and Lactation on Offspring Brain Lipid Development. *J. Neurochem*., 27(6): 1361-1365.

D'Agostino R.B., Hamman R.F., Karter A.J., Mykkanen L., Wagenknecht L.E., Haffner S.M. (2004). Cardiovascular Disease Risk Factors Predict the Development of Type 2 Diabetes The Insulin Resistance Atherosclerosis Study. *Diabetes Care*, 27(9): 2234-2240.

Denko C.W., Purser D.B., Johnson, R.M. (1970). Amino acid composition of serum albumin in normal individuals and in patients with rheumatoid arthritis. *Clinical chemistry*, 16(1): 54-57.

Dimitriadis G., Mitrou P., Lambadiari V., Maratou E., Raptis S.A. (2011). Insulin effects in muscle and adipose tissue. *Diabetes Res. Clin. Pract.*, 93: S52-S59.

Donald P., Pitts G.C., Pohl S.L. (1981). Body Weight and Composition in Laboratory Rats: Effects of Diets with High or Low Protein Concentrations. *Science,* 211(4478): 185-186.

Du F., Higginbotham A.D., White D.B. (2000). Food Intake, Energy Balance and Serum Leptin Concentrations in Rats Fed Low-Protein Diets. *J. Nutr.*, 130(3): 514-521.

El-Khoury A.E., Pereira P.C., Borgonha S., Basile-Filho A., Beaumier L., Wang S.Y., Metges C.C., Ajami A.M., Young V.R. (2000). Twenty-four-hour oral tracer studies with L-[1–13C]lysine at a low (15 mg/kg/d) and intermediate (29 mg/kg/d) lysine intake in healthy adults. *Am. J. Clin. Nut.*, 72(1): 122–30.

FAO/WHO/UNU. (1985). Energy and protein requirements. Report of a joint FAO/WHO/UNU Expert Consultation. Geneva: World Health Organization.

FAO/WHO/UNU. (2007). Protein and amino acid requirements in human nutrition. Report. Geneva: World Health Organization.

Fernandez M.L., Wilson T.A., Conde K., Vergara-Jimenez M., Nicolosi R.J. (1999). Hamsters and guinea pigs differ in their plasma lipoprotein cholesterol distribution when fed diets varying in animal protein, soluble fiber, or cholesterol content. *J. Nutr.*, 129(7): 1323–1332.

Food and Agriculture Organization of the United Nations. FAO Statistics Division 2011. Food Balance Sheet. Website Accessed on 1 March, 2016.

Food and Nutrition Board, Institute of Medicine (2002/2005). DRI report for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein and Amino Acids.

Garg A. (1996). Insulin resistance in the pathogenesis of dyslipidemia. *Diabetes Care*, 19(4): 387-389.

Gietzen D.W., Shuzhen H., Tracy G.A. (2007). Mechanisms of Food Intake Repression in Indispensible Amino Acid Deficiency. *Annu. Rev. Nutr.*, 27: 63-78.

Godfrey K., Robinson S., Barker D.J.P., Osmond C., Cox V. (1996). Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. *BMJ,* 312(7082): 410-414.

Graham G.G., Maclean Jr. W.C., De Romaň G.L. (1981). Prolonged Consumption by Infants of Wheat-Based Diets With and Without Casein or Lysine Supplementation. *J. Nutr*., 111(11): 1917- 1927.

Gutiérrez O.M. (2013). The Connection between Dietary Phosphorus, Cardiovascular Disease, and Mortality: Where We Stand and What We Need to Know. *Adv. Nutr.,* 4(6): 723–729.

Guzman J.J., Luis A.G., Martinez G.D., Perez F.X., Mascorro G.F., Inzunza G.R. (2013). Effect of lysine addition on growth of black iguana (Ctenosaura pectinata). *Zoo Biol.,* 32(3): 277-280.

Harland B.F. and Harland J. 1980. Fermentation reduction of phytate in rye, white and whole wheat breads. *Cereal Chem.* 57(3): 226-229.

Harper A.E., Benevenga N.J., Wohlheuter R.M. (1970). Effects of ingestion of disproportionate amounts of amino acid. *Physiol. Rev.,* 50(3): 428-558.

Harper A.E., Winje M.E., Benton D.A., Elvehjem C.A. (1955). Effect of amino acid supplements on growth and fat deposition in the livers of rats fed polished rice. *J. Nutr.,* 56(2): 187-198.

Hazim J., Hlais S., Ghattas H., Shatila D., Bassil M., Obeid O. (2014). Phosphorus supplement alters postprandial lipemia of healthy male subjects: a pilot cross-over trial. *Lipids Health Dis.,* 13:109.

Henry Y., Gueguen L., Rerat A. (1979). Influence of the level of dietary phosphorus on the voluntary intake of energy and metabolic utilization of nutrients in the growing rat. *Br. J. Nutr.*, 42(1): 127-137.

Herzeberg G.R. and Rogerson M. (1984). Hepatic lipogenesis in young rats given proteins of different quality. *Br. J. Nutr.,* 52(1): 131-137.

Hlais S., Reslan D.R., Sarieddine H.K., Nasreddine L., Taan G., Azar S., Obeid O.A. (2012). Effect of lysine, vitamin B(6), and carnitine supplementation on the lipid profile of male patients with hypertriglyceridemia: A 12-week, open-label, randomized, placebo-controlled trial. *Clin. Ther*., 34(8): 1674–1682.

Hrupka B.J., Lin Y., Gietzen D.W. Rogers Q.R. (1999). Lysine Deficiency Alters Diet Selection without Depressing Food Intake in Rats. *J. Nutr.,* 129(2): 424–430.

Hussain T., Abbas S., Khan M.A., Scrimshaw N.S. (2004). Lysine fortification of wheat flour improves selected indices of the nutritional status of predominantly cereal-eating families in Pakistan. *Food Nutr. Bull.,* 25(2): 114-122

Hoffman J.R. and Falvo M.J. (2004). Protein – Which is Best? *J. Sports Sci. Med.,* 3(3): 118-130

Institute of Medicine, Food and Nutrition Board. (1997). Dietary Reference Intakes for Calcium, Phosphorus, Magnesium, Vitamin D, and Fluoride. National Academy Press. Washington, DC.

Jansen G.R. and Chase P. (1976). Effect of Feeding Lysine and Threonine Fortified Bread During Gestation and Lactation on Growth of the Offspring in Rats. *J. Nutr.*, 106(1): 33-40.

Jenkins D.J.A, Kendall C.W.C., Vidgen E., Augustin L.S.A., Van Erk M., Geelen A., Parker T., Faulkner D.,Vuksan V., Josse R.G., Leiter L.A., Connelly P.W. (2001). High-protein diets in hyperlipidemia: effect of wheat gluten on serum lipids, uric acid, and renal function. *Am. J. Clin. Nutr.*, 74(1): 57–63.

Kalantar-Zadeh K., Gutekunst L., Mehrotra R., Kovesdy C.P., Bross R., Shinaberger C.S., ... & Kopple, J. D. (2010). Understanding sources of dietary phosphorus in the treatment of patients with chronic kidney disease. *Clin. J. Am. Soc. Nephrol.*, *5*(3): 519-530.

Khattab M., El-Khoury D., Azar S., Mattar M., & Obeid O. (2011). Effect of phosphorus on the oral glucose tolerance test. *Proc. Nutr. Soc.*, 70(OCE3): E60.

Khan L. and Bamji M.S. (1979). Tissue Carnitine Deficiency due to Dietary Lysine Deficiency: Triglyceride Accumulation and Concomitant Impairment in Fatty Acid Oxidation. *J. Nutr.*, 109(1): 24-31.

Krajčovičoá-Kudláčková M., Šimončič R., Béderová A., Babinská K., Béder I. (2000). Correlation of Carnitine Levels to Methionie and Lysine Intake. *Physiol. Res*., 49(3): 399-402.

Krauss R.M., Eckel R.H., Howard B., Appel L.J., Daniels S.R., Deckelbaum R.J., ... & Bazzarre T.L. (2000). AHA dietary guidelines revision 2000: a statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation*, 102(18): 2284-2299.

Kritchevsky D., Tepper S.A., Czarnecki S.K., Klurfeld D.M., Story J.A. (1981). Experimental atherosclerosis in rabbits fed cholesterol-free diets. Part 9. Beef protein and textured vegetable protein. *Atherosclerosis*, 39(2): 169–175.

Kroeckel S., Dietz C., Schulz C., Susenbeth A. (2013). Effect of diet composition and lysine supply on growth and body composition in juvenile turbot (Psetta maxima). *Animal Nutr.*, 67(4): 330–345.

Kumar V., Sinha A.K., Makkar H.P., Becker K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food Chem.*, 120(4): 945-959.

Kurpad A.V., Raj T., El-Khoury A., Beaumier L., Kuriyan R., Srivatsa A., Borgonha S., Selvaraj A., Regan M.M., Young V.R. (2001). Lysine requirements of healthy adult Indian subjects, measured by an indicator amino acid balance technique. *Am. J. Clin. Nut.*, 73(5): 900–907.

Kurpad A.V., Regan M.M., Raj T., El-Khoury A., Kuriyan R., Vaz M., Chandakudlu D., Venkataswamy V.G., Borgonha S., Young V.R. (2002). Lysine requirements of healthy adult Indian subjects receiving long-term feeding, measured with a 24-h indicator amino acid oxidation and balance technique. *Am. J. Clin. Nut.*, 76(2): 404–412.

Kurpad A.V., Regan M.M., Raj T., Vasudevan J., Kuriyan R., Gnanou J., Young V.R. (2003). Lysine requirements of chronically undernourished adult Indian men, measured by a 24-h indicator amino acid oxidation and balance technique. *Am. J. Clin. Nut.*, 77(1): 101–108.

Leung P.M., Rogers Q.R., Harper A.E. (1968). Effect of amino acid imbalance on dietary choice in the rat. *J. Nutr., 95*(3): 483-492.

Li X.Y., Tang L., Hu K., Liu Y., Jiang W.D., Jiang J., Wu P., Chen G.F., Li S.H., Kuang S.Y. Feng L., Zhou X.Q. (2014). Effect of dietary lysine on growth, intestinal enzymes activities and antioxidant status of sub-adult grass carp (Ctenopharyngodon idella). *Fish Physiol. Biochem.*, 40(3): 659-671.

Lippi G., Montagnana M., Salvagno G.L., Targher G., & Guidi G.C. (2009). Relationship between serum phosphate and cardiovascular risk factors in a large cohort of adult outpatients. *Diabetes Res. Clin. Pract.*, 84(1): e3-e5.

Lopez H.W., Krespine V., Guy C., Messager A., Demigne C., and Remesy C. 2001. Prolonged Fermentation of Whole Wheat Sourdough Reduces Phytate Level and Increases Soluble Magnesium. *J. Agric. Food Chem.* 49(5): 2657-2662.

Mahgoub S.E.O. and Elhag S.A. 1998. Effect of milling, soaking, malting, heat-treatment and fermentation on phytate level of four Sudanese sorghum cultivars. *Food Chem.* 61(1): 77-80.

Malta A., De Oliveira J.C., Da Silva Ribeiro T.A., To´folo L.P., Barella L.F., Prates K.V., Miranda R.A., Elmhiri G., Da Silva Franco C.C., Agostinho A.R., Trombini A.B., Pavanello A., Gravena C., Abdennebi-Najar L., De Freitas Mathias P.C. (2014). Low-protein diet in adult male rats has long-term effects on metabolism. *J. Endocrinol.*, 221(2): 285–295.

Mattar M.A. and Obeid O.A. (2010). Energy efficiency of rats maintained on a diet with varied casein to gluten ratios. *Curr. Top. Nutraceutical Res.*, 8: 95–102.

McMurray R.G. and Hackney A.C. (2005). Interactions of metabolic hormones, adipose tissue and exercise. *Sports Medicine*, 35(5): 393-412.

Mercer L.P., David F.W., Ramlet J. S. (1981). Control of Food Intake in the Rat by Dietary Protein Concentration. *J. Nutr.*, 111(6): 1117-1123.

Mikkelsen P.B., Toubro S., Astrup A. (2000). Effect of fat-reduced diets on 24 h energy expenditure: comparisons between animal protein, vegetable protein, and carbohydrate. *Am. J. Clin. Nutr.*, 72(5): 1135–1141.

Millet S. and Aluwé M. (2014). Compensatory growth response and carcass quality after a period of lysine restriction in lean meat type barrows. *Arch. Anim. Nutr.*, 68(1): 16–28.

Millward J. (1997). Human Amino Acid Requirements. *J. Nutr.* 127(9): 1842–1846.

Millward D.J., Fereday A., Gibson N.R., Cox M.C., Pacy P.J. (2002). Efficiency of utilization of wheat and milk protein in healthy adults and apparent lysine requirements determined by a single-meal [1–13C]leucine balance protocol. *Am. J. Clin. Nut.*, 76(6): 1326 –34.

Millward D.J., Fereday A., Gibson N.R., Pacy P.J. (2000). Human adult amino acid requirements: [1–13C]leucine balance evaluation of the efficiency of utilization and apparent requirements for wheat protein and lysine compared with those for milk protein in healthy adults. *Am. J. Clin. Nut.*, 72(1): 112–21.

Moe S.M., Zidehsarai M.P., Chambers M.A., Jackman L.A., Radcliffe J.S., Trevino L.L., Donahue S.E., Asplin J.R. (2011). Vegetarian Compared with Meat Dietary Protein Source and Phosphorus Homeostasis in Chronic Kidney Disease. *Clin. J. Am. Soc. Nephrol.,* 6: 257–264.

Montonen J., Knekt P., Ja¨rvinen R., Aromaa A., Reunanen A. (2003). Whole-grain and fiber intake and the incidence of type 2 diabetes. *Am. J. Clin. Nut.*, 77(3): 622–629.

Munaver S.M. and Harper A.E. (1959). Amino acid balance and imbalance. II. Dietary levels of protein and lysine requirement. *J. Nutr.*, 69: 58-64.

Nagata C., Takatsuka N., Kurisu Y., Shimizu H. (1998). Decreased serum total cholesterol concentration is associated with high intake of soy products in Japanese men and women. *J. Nutr.*, 128(2): 209–213.

Neuberger A. and Webster T.A. (1945). The Lysine Requirements of the Adult Rat. *Biochem. J.*, 39(2): 200-202.

Obeid O. (2013). Low phosphorus status might contribute to the onset of obesity. *Obes. Rev., 14*(8): 659-664.

Obeid O.A., Hachem D.H., Ayoub, J.J. (2014). Refeeding and metabolic syndromes: two sides of the same coin. *Nutr. Diabetes*, 4(6): e120.

Obeid O., Khatib L., Batal M., et al. (2008). Established and suspected biomarkers of cardiovascular disease risk in pre-menopausal Lebanese women. *Ecol. Food Nutr.*, 47: 298–311.

Ousterhout L.E. (1960). Survival time and biochemical changes in chicks fed diets lacking different essential amino acids. *J. Nutr.*, 70: 226-34.

Park W., Kim B.S., Lee J.E., Huh J.K., Kim B.J., Sung K.C., ..., Kim S.W. (2009). Serum phosphate levels and the risk of cardiovascular disease and metabolic syndrome: a double-edged sword. *Diabetes Res. Clin. Pract.*, 83(1): 119-125.

Pastoor F.J.H., Opitz R., Van 'T Klooster A.TH., Beynen A.C. (1995). Dietary phosphorus restriction to half the minimum required amount slightly reduces weight gain and length of tibia, but sustains femur mineralization and prevents nephrocalcinosis in female kittens. *Br. J. Nutr.* 74(1): 85-100.

Peng Y., Meliza L.L., Vavich M.G., Kemmeher A.R. (1974). Changes in Food Intake and Nitrogen Metabolism of Rats While Adapting to a Low or High Protein Diet. *J. Nutr.*, 104(8): 1008-1017.

Potter J.D. (2000). Your mother was right: eat your vegetables. *Asia. Pac. J. Clin. Nutr.*, 9(1): S10–S12.

Radcliffe J.D. and Webster A.J.F. (1976). Regulation of food intake during growth in fatty and lean female Zucker rats given diets of different protein content. *Br. J. Nutr.*, 36(3): 457-469.

Ravindran V., Ravindran G., and Sivalogan S. 1994. Total and phytate phosphorus contents of various foods and feedstuffs of plant origin. *Food Chem.* 50: 133-136.

Scrimshaw N.S., Taylor Y., Young V.R. (1973). Lysine supplementation of wheat gluten at adequate and restricted energy intakes in young men. *Am. J. Clin. Nut.*, 26(9): 965-972.

Shills M.E., Olson J.A., Shike M., Ross A.C. (2005). Modern Nutrition in Health and Disease (10^{th} edition), Lippincott Williams and Wilkins. Chapter 10.

Sidransky H. and Baba T. (1960). Chemical pathology of acute amino acid deficiencies. III. Morphologic and biochemical changes in young rats fed valine- or lysine-devoid diets. *J. Nutr.*, 70: 463-83.

Singh P.N., Sabaté J., Fraser, G.E. (2003). Does low meat consumption increase life expectancy in humans? *Am. J. Clin. Nutr.*, 78(3 Suppl): 526S–32S.

Sluijs I., Beulens J.W.J., Van Der A D.L., Spijkerman A.M.W., Grobbee D.E., Van Der Schouw Y.T. (2010). Dietary Intake of Total, Animal, and Vegetable Protein and Risk of Type 2 Diabetes in the European Prospective Investigation into Cancer and Nutrition (EPIC)-NL Study. *Diabetes Care,* 33(1): 43-48.

Stockland W.L., Meade R.J., Melliere A.L. (1970). Lysine Requirement of the Growing Rat: Plasma free Lysine as a Response Criterion. *J. Nutr.*, 100(8): 925-934.

Sulochana K.N., Lakshmi S., Punitham R., Arokiasamy T., Sukumar B., Ramakrishnan S. (2002). Effect of oral supplementation of free amino acids in type 2 diabetic patients -- a pilot clinical trial. *Med. Sci. Monit*., 8(3): CR132–C137.

Swick R.W. and Gribskov A.L. (1983). The Effect of Dietary Protein Levels on Diet-Induced Thermogenesis in the Rat. *J. Nutr.*, 113(11): 2289-2294.

Symeou V., Leinonen I., Kyriazakis I. (2014). Modelling phosphorus intake, digestion, retention and excretion in growing and finishing pigs: model description. *Animal,* 8(10): 1612–1621.

Takeda E., Yamamoto H., Yamanaka-Okumura H., Taketani Y. (2014). Increasing Dietary Phosphorus Intake from Food Additives: Potential for Negative Impact on Bone Health. *Adv. Nutr.*, 5(1): 92–97.

Tanaka S., Yamamoto H., Nakahashi O., Kagawa T., Ishiguro M., Masuda M., ... & Takeda E. (2013). Dietary phosphate restriction induces hepatic lipid accumulation through dysregulation of cholesterol metabolism in mice. *Nutri. Res.*, *33*(7): 586-593.

Tang Q., Wang C., Xie C., Jin J., Huang Y. (2012). Dietary Available Phosphorus Affected Growth Performance, Body Composition, and Hepatic Antioxidant Property of Juvenile Yellow Catfish Pelteobagrus fulvidraco. *Scientific World Journal*, 2012(987570).

Tanphaichitr V., Zaklama M.S., Broquist H.P. (1976). Dietary lysine and carnitine: relation to growth and fatty livers in rats. *J. Nutr*., 106(1): 111–117.

Tein I., Bukovac S.W., Xie Z.W. (1996). Characterization of the human plasmalemmal carnitine transporter in cultured skin fibroblasts. *Arch. Biochem. Biophys*., 329(2): 145-55.

Teixeira S.R., Potter S.M., Weigel R., Hannum S., Erdman J.W., Hasler C.M. (2000). Effects of feeding 4 levels of soy protein for 3 and 6 wk on blood lipids and apolipoproteins in moderately hypercholesterolemic men. *Am. J. Clin. Nutr*., 71(5): 1077–1084.

Terpstra A.H.M., Holmes J.C., Nicolosi R.J. (1991). The hypocholesterolemic effect of dietary soybean protein vs. casein in hamsters fed cholesterol-free or cholesterolenriched semipurified diets. *J. Nutr.*, 121(7): 944–947.

Thalacker-Mercer A.E., Fleet J.C., Craig B.A., Carnell N.S., and Campbell W.W. (2007). Inadequate protein intake affects skeletal muscle transcript profiles in older humans. *Am. J. Clin. Nut.*, 85(5): 1344–1352.

Tomé D. and Bos C. (2007). Lysine Requirement through the Human Life Cycle. *J. Nutr.*, 137(6 Suppl 2): 1642S-1645S.

Uribarri J. and Calvo M.S. (2013). Dietary Phosphorus Excess: A Risk Factor in Chronic Bone, Kidney, and Cardiovascular Disease? *Adv. Nutr*., 4(5): 542–544.

U.S. Department of Agriculture, Agricultural Research Service. (2016). USDA National Nutrient Database for Standard Reference, Release 28. Nutrient List Home Page, https://ndb.nal.usda.gov/ndb/nutrients/index

Visanuvimola L. and Bertramb S.M. (2010). How dietary phosphorus availability during development influences condition and life history traits of the cricket, Acheta domesticus. *J. Insect Sci.*, 11(63): 1536-2442.

Westerterp-Plantenga M.S. (2003). The significance of protein in food intake and body weight regulation. *Curr. Opin. Clin. Nutr. Metab. Care*, 6(6): 635–638.

Walton M.J., Cowey C.B., Adron J.W. (1984). The effect of dietary lysine levels on growth and metabolism of rainbow trout (Sulmo gairdnerz). *Br. J. Nutr.*, 52(1): 115-122.

White D.B., Porter M.H., Martin R.J. (2000). Effects of age on the feeding response to moderately low dietary protein in rats. *Physiol. Behav.*, 68(5): 673–681.

Yamashita K. and Ashida K. (1969). Lysine Metabolism in Rats Fed Lysine-free Diet. *J. Nut.*, 99(3): 267-273.

Young V.R., Bier D.M., Pellett P.L. (1989). A theoretical basis for increasing current estimates of the amino acid requirements in adult man, with experimental support. *Am. J. Clin. Nut.*, 50(1): 80-92.

Young V.R., and Marchini J.S. (1990). Mechanisms and nutritional significance of metabolic responses to altered intakes of protein and amino acids, with reference to nutritional adaptation in humans. *Am. J. Clin. Nutr.*, *51*(2): 270-289.

Zhao W., Zhai F., Zhang D., An Y., Liu Y., He Y., Ge K., Scrimshaw N.S. (2004). Lysine-fortified wheat flour improves the nutritional and immunological status of wheat-eating families in northern China. *Food Nutr. Bull.*, 25(2): 123-129.