AMERICAN UNIVERSITY OF BEIRUT

LOW-QUALITY PROTEIN MODULATES INFLAMMATORY MARKERS AND THE RESPONSE TO LIPOPOLYSACCHARIDE INSULT: THE CASE OF LYSINE

by
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ABSTRACT
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Title: Low-quality protein modulates inflammatory markers and the response to lipopolysaccharide insult: The case of lysine.

The relationship between non-communicable diseases (NCDs) and eating behavior has long been an area of interest. The literature largely attributes the association between NCDs and nutrition to a surplus of food and energy. However, a population observation brought to our attention a dietary factor that may explain the increase in the prevalence of NCDs among the least advantaged communities. Over the last couple of decades, the world has witnessed an increase in the prevalence of NCDs that has paralleled a major nutrition transition characterized by very high cereal intake among populations with low socioeconomic status (SES). Cereals contain low amounts of proteins, which naturally lack lysine, an essential amino acid, in adequate quantities. Lysine is a main component of the amino acid composition of both serum albumin and c-reactive protein (CRP), which concentrations were shown to change among people with low-grade inflammation, a hallmark of NCDs. Hence, it is thought that lysine deficiency might be a confounding factor explaining the association between nutrition and NCDs.

In this work, we aim to study the effect of varied levels of lysine deficiency (60% and 30% deficiency) on body composition, inflammatory profile, and behavior of rats in the absence or presence of an inflammatory insult [lipopolysaccharide (LPS)]. For this purpose, two experiments were designed on five-week-old male Sprague Dawley rats to meet the objective of the work. Both experiments were identical and only differed in the level of lysine deficiency. In experiment 1, rats were randomly distributed into four groups: 1) control diet, 2) control diet+LPS, 3) 60% lysine-deficient diet, and 4) 60% lysine-deficient diet+LPS. Experiment 1 was followed by experiment 2, which held three experimental groups: 1) control diet, 2) 30% lysine-deficient diet, and 3) 30% lysine-deficient diet+LPS. Rats were only allowed their experimental diets for four weeks, during which LPS (50 µg/kg) or saline injections were administered intraperitoneally three times per week.

The study showed that 60% lysine deficiency blunted growth and body compartments development, decreased albumin production, and raised hepatic c-reactive protein (CRP) expression, independently of interleukins 6 and 1β, the main precursors of CRP. Also, the 60% insufficient levels of lysine in the diet triggered hyperactivity and anxiety-like behavior. On the other hand, a marginal lysine deficiency of 30%, did not

yield drastic changes in rats, suggesting dose-dependent alterations associated with the level of deficiency. LPS administration exacerbated outcomes related to body composition, metabolism, and behavior caused by 60% lysine deficiency but surprisingly did not worsen albumin and CRP expressions. This work presents evidence of varied physiological changes associated with insufficient amounts of lysine intake that can potentially increase risk factors for diseases. Thus, the increment in NCDs among the low SES populations, who heavily rely on cereals as a main source of protein may be blamed on low lysine availability in diets.

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ABBREVIATIONS

-	Minus
%	Percent
/	Per
+	Plus
÷	Divide
<	Less than
>	Greater than
Δ	Change in
x	Times
5-HT4	Serotonin to its receptor 4
ADHD	attention-deficit/hyperactivity disorder
ANOVA	Analysis of variance
AUB	American University of Beirut
AUC	Area under the curve
CDC	Centers for Disease Control and Prevention
cDNA	Complementary DNA
CHD	Coronary heart diseases
CRP	C-reactive protein
CVD	Cardio vascular diseases
d	Day
dl	Deciliter
DRI	Dietary reference intake
EAR	Estimated average requirements
EDTA	Ethylenediaminetetraacetic acid
EE	Energy expenditure
EEf	Energy efficiency
FAO	Food and Agriculture Organization
FGF21	Fibroblast growth factor 21
g	Gram
Glu	Glucose
HDL	High-density lipoprotein
HOMA	Homeostatic model assessment for insulin resistance
HPLC	High-performance liquid chromatography
hs-CRP	High sensitivity CRP
Ig	Immunoglobulin
IL-1β	Interleukin 1 β
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
INF-γ	Interferon γ
Ins	Insulin
IPGTT	Intraperitoneal glucose tolerance test
IRE1	Inositol-requiring protein 1
kg	Kilogram
L	Liter
LDL	Low-density lipoprotein
LNAA	Large neutral amino acid

MCP-1	Monocyte chemoattractant protein-1
mg	Milligram
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NEAT	Non-Exercise Activity Thermogenesis
OFT	Open field test
pH	Potential of hydrogen
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SES	Socioeconomic status
TG	Triglycerides
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
WHO	World Health Organization
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

This work is dedicated to my beloved father, Abdallah El Mallah, who passed away on January 30, 2022. I owe it all to him.

CHAPTER I

LITERATURE REVIEW

A. Non-communicable diseases

1. Overview

Non-communicable diseases (NCDs) are the world's leading cause of death and pose an emerging health threat at a global level (CDC, 2021). Once thought of as "diseases of the rich", NCDs are now known to predominantly afflict people in low and middle-income countries (WHO, 2011). In fact, these diseases kill 41 million yearly, among which 77% live in low- and middle-income countries (WHO, 2022).

Recent data highlighted the tight association found between low socioeconomic status (SES), cardiovascular diseases (CVDs), and some types of cancer (Williams et al., 2018), while others showed a heightened vulnerability to developing type 2 diabetes and chronic obstructive pulmonary disease (Sommer et al., 2015). In addition, population-based studies have described the relationship between an increased risk of mood disorders, anxiety, substance use disorder (Sareen et al., 2011), and depression on the one hand and low income on the other hand (Lorant et al., 2003).

2. The underlying conditions

It is no surprise to mention that low-grade inflammation precedes the onset of various diseases. Compiling evidence suggests a critical role for chronic low-grade inflammation in the pathogenesis and etiology of obesity, insulin resistance and type 2 diabetes (Wu & Ballantyne, 2020), coronary heart diseases (CHDs) (Arnold et al.,

2021), cancers (Greten & Grivennikov, 2019), depression and anxiety (Ye et al., 2021), etc. Interestingly, C-reactive protein (CRP) or high-sensitivity CRP (hs-CRP) is a hallmark of low-grade systemic inflammation, and indeed, studies have reported an increase in its levels among populations with NCDs. For instance, a population-based cross-sectional study on 3,918 subjects from 13 villages in China confirmed a median (interquartile range) of 0.95 (0.40–2.36) mg/l of CRP in non-diabetic subjects as compared to 1.92 mg/l (0.71–4.27) in subjects with diabetes, and the difference was highly statistically significant (Wen et al., 2010). Likewise, a subsample analysis in a cohort study confirmed the increased risk of CVD incidents among men with high CRP (hazard ratios (HR) of 2.39), making this marker a predictor of CVD events (Tuomisto et al., 2006). Also, a comprehensive systematic review that investigated the relationship of inflammatory markers with depression in both clinical and community samples concluded a positive association between this mental disorder, CRP, and some interleukins (IL) (Howren et al., 2009).

3. Inflammation and low SES

Substantial documentation affirms systemic inflammation among people with low SES. In a meta-analysis, data from 111,156 individuals (from 43 studies) indicated that people with lower SES had higher levels of CRP (Muscatell et al., 2020). Also, in childhood and adolescence, Milaniak & Jaffee displayed a small yet significant inverse association between SES and CRP (Milaniak & Jaffee, 2019). In support, Liu *et al.* found that the most advantaged children and adults had 25% lower CRP levels than the least advantaged ones (Liu et al., 2017). This result was similar to the findings from

NHANES data on children (n=13,165) (Schmeer & Yoon, 2016), and supporting conclusions are present in the literature (Lam et al., 2021; Surachman et al., 2020).

4. NCDs-associated factors

NCDs result from a combination of genetic, environmental, and behavioral factors (WHO, 2022), making their prevention and management complex to address. Interventions to reduce incidence of NCDs are directed toward changing behavioral factors, such as physical inactivity, smoking, alcohol abuse, eating unhealthy diets, etc. (WHO, 2022), that were demonstrated to increase the risk of diseases. For instance, a sedentary lifestyle, as indicated in a study by Lee *et al.*, raises the odds of developing CHD by 54%, type 2 diabetes by 20%, breast cancer by 35%, and colon cancer by 37% (Lee et al., 2012). On the other hand, tobacco consumption is responsible for 1 in 6 deaths by NCDs (NCD Alliance, 2022), while alcohol is detrimentally correlated with many adverse cardiovascular outcomes (Patra et al., 2010; Taylor et al., 2009).

Unhealthy diets are one of the major drivers of NCDs. The world has witnessed a drastic change in nutrition over the last decades, known as the westernization of dietary patterns, characterized by a diet high in refined carbohydrates, fats, and processed foods (Popkin & Gordon-Larsen, 2004). This transition was paralleled by the increased incidence and prevalence of NCDs and their associated deaths (Martínez-González & Martín-Calvo, 2013; Oggioni et al., 2014). In their study, Pressler *et al.* assessed several health outcomes of four different populations before and after dietary transitions. The study populations were reported to be

predominantly leaner and had lower prevalences of diabetes and CHD before food westernization (Pressler et al., 2022).

5. Nutrition factor and low SES

The same time frame that witnessed diet westernization among developing countries has observed an exponential upsurge in the death caused by NCDs among the low and middle-income populations. While lack of exercise, smoking, high body mass index (BMI), and alcohol consumption are responsible for a large share of the global disease burden linked with SES (Ezzati & Riboli, 2013), they do not entirely account for these associations. Here comes a particular emphasis on the nutrition and dietary habits of low SES populations in the context of global westernization, which has kept low SES people at high risk of NCDs.

The intake of animal proteins was shown to decrease with low income and be replaced with vegetable proteins and carbohydrates (Popkin & Gordon-Larsen, 2004). Indeed, due to the high expense of animal-based products, low SES communities procure most of their protein and energy from plant sources, namely cereals (Young & Pellett, 1994), keeping the consumption of refined carbohydrate and vegetable fats high (Popkin & Gordon-Larsen, 2004). In fact, plant protein makes up more than 80% of the total protein intake among the least developed populations (Young & Pellett, 1994).

B. Plant proteins

Including plant-based protein within a healthy and balanced diet has been proven to yield paramount benefits (Dinu et al., 2017). However, increasing these types of proteins should not happen at the expense of covering all the essential amino acids.

While protein quality in plant-based diets has long been a matter of discussion due to the lack of one or more essential amino acids (Hoffman & Falvo, 2004), animal products are known to contain a complete profile of indispensable amino acids. Hence, substituting meats with grains or legumes should be framed within the concept of protein complementation, in which protein sources are mixed to supplement the limited content of amino acids and improve the quality of the protein (Hoffman & Falvo, 2004). In animals, adding 5% casein (animal-based protein) to the wheat gluten diet was found to improve the quality of dietary protein and prevent the decline in weight gain, food intake, and feed efficiency (Mattar & Obeid, 2010). On the other hand, exclusively consuming incomplete gluten proteins, Sprague Dawley rats had defects in growth, development, and energy efficiency (Ragi et al., 2019).

Unfortunately, some populations, especially people of low SES, rely highly on cereals, especially wheat, as a primary source of protein and energy (Rosegrant et al., 1999). Based on data from Food and Agriculture Organization (FAO), at the global level, wheat has been the cereal the most supplied since 1970, as shown in Figure 1 (FAO, 2021).

Proteins from cereals (wheat, rice, and maize) lack lysine (Bos et al., 2005), which makes this essential amino acid the first limiting amino acid in cereals (Vasal, 2004) and puts people who exclusively depend on cereals at risk of lysine deficiency. Indeed, lysine intake among vegans was reported to be around half that of omnivores (Krajcovicová-Kudlácková et al., 2000).

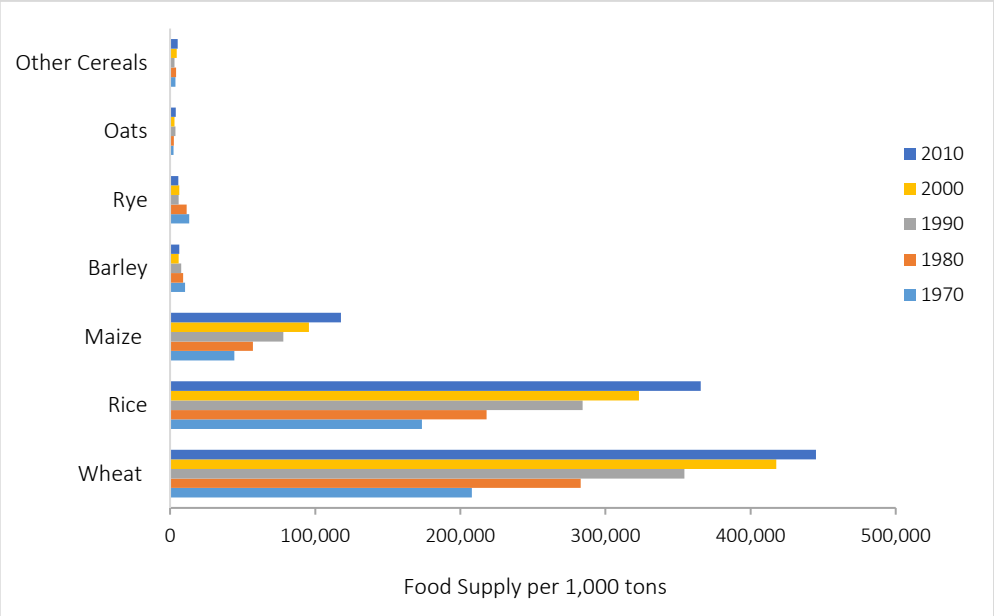

Figure 1. Cereals supplies in the world from 1970 to 2010
The world's food supply (per 1000 tons) of wheat, rice, maize, barley, rye, oats, and others was probed using the Food Balances Sheets using the old methodology and population of the FAOSTAT from the year 1970 to 2010 (FAO, 2021).

C. Lysine

1. Definition and food sources

Lysine is a basic amino acid that is positively charged at physiological pH (Engelking, 2015). It is classified as essential amino acid, meaning that it cannot be synthesized by the body and must be obtained from food in adequate amounts. Table 1 displays lysine content in different food items. Animal products such as meat, poultry, fish, and dairy have high lysine content, more than 2.7 fold the amount found in plant-based products, among which the lowest ratios are held in cereals with around 31 mg of lysine per gram of protein (Young & Pellett, 1994). For instance, wheat protein shares the lowest value of lysine content with 22–35 mg/g compared to rice (30–36 mg/g) and maize (28–42 mg/g) (Millward, 1999).

Table 1. Selected food sources of lysine

Food item	Lysine	
	mg/100g	mg/g protein
Animal products		
Parmesan cheese	3,306	92.3
Braised beef steak	3,053	84.6
Braised lamb	3,018	88.2
Braised veal	2,944	82.5
Roasted turkey	2,907	94.1
Fried chicken breast	2,836	84.9
Broiled pork	2,799	90
Smoked red salmon	2,740	77.8
Gruyere cheese	2,710	90.9
Canned tuna (drained)	2,675	91.9
Dry milk	2,087	79.4
Animal fats	0	0
Plant products		
Oats	701	41.5
Rye	605	40.8
Barley	369	37.3
Whole-grain wheat flour	378	27.6
White rice	339	36.2
Whole-wheat pita bread	265	27.0
White wheat flour	228	22.1
White pita bread	219	24.1
White rice flour	207	35.1
Raw okra/asparagus/cauliflower/white mushrooms	81-107	34.2-45.2
Raw onions/peppers/pumpkin/tomatoes/cabbages/eggplant	39-47	34.2-41.2
Raw Blueberries/Apples/Grapes/Plums/ Nectarines/Pears/Peaches/Figs/Apricots	13-30	23.2-53.6
Vegetable oils	0	0

Data are retrieved and adapted from Nutrition Data (Nutrition Data, 2014).

2. Requirements

The need for lysine has been underestimated for years. This is clearly shown through the increase in the recommended daily intake. Back in 1954, Rose *et al.*, using the nitrogen balance technique, suggested an amount of 800 mg/d, equivalent to 12 mg/kg/d, as the minimal daily lysine requirement in men (Rose et al., 1955). These values were adopted by FAO/WHO nutrition meeting in 1973 and remained valid after the meeting in 1985 (FAO/WHO, 1973; FAO/WHO, 1985).

Studying lysine kinetics among healthy young men and tracing their lysine intake and amino acid concentrations, Meredith *et al.* concluded that a plasma balance was achieved when subjects consumed more than 32 mg/kg/d of lysine (Meredith et al., 1986). The authors criticized the previous work stating that low recommendations resulted from a poor study design, in which men were on high energy intake and positive nitrogen balance. A series of subsequent studies presented a wide range from 17 to more than 41 mg/kg of lysine daily (Duncan et al., 1996; El-Khoury et al., 2000; Millward et al., 2002; Rand & Young, 1999; Zello et al., 1993).

In its latest nutrition meeting in 2007, the joint committee of FAO and WHO adjusted lysine recommendations to 30 mg/kg/d for adults, equivalent to 45 mg/g of protein (FAO/WHO/UNU, 2007). Thus, a 70-kilogram person would need around 959 to 1,151 g of white pita bread to meet the daily needs of lysine. Similar values were suggested by the institute of medicine, which set the estimated average requirement (EAR) for 19 years old and older adults at 31 mg/kg/d (IOM, 2005). Requirements in younger, growing individuals are known to be higher, accounting for the increased needs related to development.

In rats, the estimated lysine requirements have also changed over the years. The National Research Council (NRC), first in 1972, recommended an intake of 9 g/kg of diet for growing and reproducing rats (NRC, 1972), and this value dropped to 7 g/kg in 1978 (NRC, 1978). In 1995, the NRC updated the requirements for laboratory rodents and suggested an intake of 9.2 g/kg of diet in growing and reproducing animals and 1.1 g/kg for the maintenance of non-reproducing adult rats (NRC, 1995). The discrepancies in the recommendations were attributed to the difference in the methods used for the estimations (NRC, 1995).

3. Wheat and gluten

Wheat comprises 85% of carbohydrates (80% of which is starch) and only a modest quantity of protein, 10% to 15% of the dry weight (Shewry & Hey, 2015). Gluten contains two types of proteins: glutenins and gliadins. They together form up to 80% of the total proteins in wheat (Uthayakumaran & Wrigley, 2017). The protein content and amino acid profile of wheat differ with the variety of cultivars, sharing a common low concentration of lysine, the first limiting amino acid in cereals (Siddiqi et al., 2020). In fact, an analysis of amino acid composition of wheat gluten shows a very high concentration of glutamine (2450 μmol/g) and proline (1080 μmol/g) at the expense of lysine (110 μmol/g) (Rombouts et al., 2009). This imbalanced amino acid profile accounts for the poor biological value of wheat, which is better explained in the following study. After administering 136 g of wheat protein containing around 25 g of labeled nitrogen [^{15}N], 14 healthy adults were subjected to intestinal and other specimen collection (Bos et al., 2005). This study showed that the postprandial utilization/retention of wheat proteins was lower than that of milk and legumes. Also, gluten has around 70% less “protein efficacy ratio” compared to beef and around 75% less “digestibility corrected amino acid score” compared to eggs (Hoffman & Falvo, 2004). Moreover, the lowest “digestible indispensable amino acid score” range was attributed to cereals (1% to 77%) due to their shortage in lysine content, followed by legumes and nuts (43% to 105% and 83% to 86%, respectively), and animal-based products (ranging from 80% and reaching 144%) (Adhikari et al., 2022).

4. Plasma concentration

Plasma amino acid concentrations were known to vary with physiological and pathological conditions; however, the relationship between plasma lysine levels and diseases is far from being conclusive. Aside from the branched-chain amino acids (BCAA), lysine was the only essential amino acid found to significantly increase (*p*-value=0.006) among obese subjects compared to non-obese (Takashina et al., 2016). Rats that were given a high-quality low-protein diet (0%, 5%, and 10% of total energy) had a lower plasma lysine than the control (15% protein) (Pezeshki et al., 2016). Interestingly, a subsample analysis within the Singapore Chinese Health Study identified a correlation between plasma lysine and the prevalence but not the incidence of type 2 diabetes (Lu et al., 2019). These studies raise the question of whether a defect in lysine metabolism is a cause or consequence of metabolic disturbances.

In protein energy malnutrition (PEM), 24 elderly were compared to 44 control subjects. Analysis of their plasma revealed that levels of lysine, threonine, and BCAA dropped significantly in PEM (Polge et al., 1997). However, this was not the case when older healthy people were compared to younger ones. Bancel *et al.* presented the differences in the amino acid profiles among healthy subjects of different ages and reported that lysine concentration was higher in older people (Bancel et al., 1994). This clearly states that in healthy conditions, young adults retain more lysine in their tissues and body proteins, while older people decrease retention and mobilize amino acids from the peripheral tissue, as a normal process of aging, resulting in more free circulating lysine in the blood. However, in case of deficiency and stress like in PEM, the body loses the ability to respond to diet normally; instead, it prioritizes the synthesis of blood

protein and preservation of the lean body mass integrity. This is likely to result in less lysine remaining in circulation.

5. Function

Lysine is one of the most abundant essential amino acids in body proteins (Matthews, 2020), which explains its importance in the system's normal functioning. It highly influences body metabolism as a molecule or its catabolites. If not used for protein synthesis, lysine undergoes catabolism that happens in the mitochondria, primarily in the liver, and results in the generation of different compounds, namely acetyl-CoA (Matthews, 2020), which intersects with numerous metabolic pathways (Shi & Tu, 2015). The functions of this amino acid are better demonstrated in deficiency and supplementation studies.

6. Deficiency and supplementation studies

Consuming inadequate amounts of any essential amino acids or, in other words, exclusively relying on low-quality proteins have detrimental health implications. As a compensatory mechanism in the case of deficiency, the body seeks higher quantities of deficient compounds to meet the needs; consequently, eating behaviors change accordingly. A diet selection study reported that rats chose a diet containing as little as 0.01% more lysine than their initial lysine-deficient diet (Hrupka et al., 1999). Animal studies showed that a gluten diet, naturally deficient in lysine, blunted growth, decreased food intake, and altered energy efficiency (Burns et al., 1982; Mattar & Obeid, 2010; Ragi et al., 2019). It has been shown in a study on pigs that defects in growth and development in lysine deficiency were attributed to the lack of

lysine itself, a crucial protein building block, and not to insulin, insulin growth factor 1 (IGF-1), and growth hormone (Hasan et al., 2020).

Lysine is an active component of the lean mass, notably the muscles. Lysine deficiency in growing broilers decreased tissue protein content and deposition due to the reduced protein synthesis fractional rate in the muscles (Tesseraud et al., 1996). In fact, lysine was shown to activate the mammalian target of rapamycin (mTOR) pathway via the upregulation of its phosphorylated form (Liu et al., 2022). Also, lysine addition to a low protein diet reversed the mRNA downregulation of IGF-1 and upregulation of myostatin (MSTN) and ww domain-containing e3 ubiquitin protein ligase 1 (WWP-1) in the muscle, and suppressed markers of autophagic proteolysis through the Akt/mTOR signaling pathway (Sato et al., 2015), supporting skeletal muscle development (Liu et al., 2022).

In addition, lysine is a precursor of carnitine, a compound that aids the long-chain fatty acids transport inside the mitochondria for oxidation. A wheat-based diet diminished carnitine levels in the plasma and muscles of rats and impaired palmitate oxidation in the heart while increasing the triglyceride content of the heart, skeletal muscle, and liver (Khan & Bamji, 1979). On the contrary, wheat fortification with lysine and threonine increased muscle carnitine levels and improved palmitate oxidation (Hirabayashi et al., 2006). Inline, adding lysine to chow feeding reduced lipid accumulation in the liver (Lin et al., 2014). Nevertheless, lysine does not seem to be the sole actor in this play. An interplay between amino acids was proposed to further explain the signals that lead to lipid incorporation in tissues. For instance, the increase in threonine serum levels in rats fed with a low lysine diet resulted in an accumulation of fat in muscles (Goda et al., 2021).

Furthermore, lysine was seen to improve numerous health outcomes in unconventional ways. In a randomized double-blinded controlled trial on hypertensive adults in Ghana, lysine supplementation was shown to normalize systolic blood pressure of men by decreasing the values from 146.11 ± 11.92 to 128.95 ± 10.44 mmHg (Vuvor et al., 2017). While authors attributed the normotensive effect of lysine to its central role in the modulation of neurotransmitters, this finding is yet to be elucidated.

Lysine has an important effect at the central level, hence its involvement in many disorders. High-lysine-supplemented diets have been associated with a lower risk of Alzheimer's disease (Rubey, 2010) and have been a target for Schizophrenia treatment (Wass et al., 2011). In fact, lysine is an essential precursor of de novo synthesis of glutamate, an excitatory neurotransmitter in the central nervous system (Papes et al., 2001). Also, lysine deficiency could lead to an increased serotonin release in the amygdala and a dysregulation in the noradrenaline release in the hypothalamus (Smriga et al., 2000; Smriga et al., 2002). A three-month randomized double-blind lysine fortification of wheat reported reduced chronic anxiety among some Syrian communities (Smriga et al., 2004). Likewise, oral lysine, along with arginine, alleviated traits of anxiety and reduced salivary levels of cortisol and chromogranin-A among healthy Japanese adults (Smriga et al., 2007).

When the impact of the fortification of wheat flour with lysine was assessed in Northern China, Zhao *et al.* demonstrated a significant increment in plasma levels of prealbumin, immunoglobulins G, A, and M, and complement factor 3 (Zhao et al., 2004), which reflects a better immunological status. Similar results were also shown in different other settings (Akalu et al., 2010; Hussain et al., 2004; Ghosh et al.,

2008). These findings suggest an association between lysine and immunity that can be mediated by inflammation.

D. Inflammation

1. Overview

Inflammation is the body's natural reaction to disrupted tissue homeostasis (Medzhitov, 2008). The acute inflammatory response can result from infection, injury, autoimmune or allergic reactions, etc. This short-term adaptive reaction is an important first line of defense; its ultimate purpose is to rule out or hinder the underlying cause and restore the cell/tissue homeostasis. While inflammation is perceived as a favorable reaction to reestablish normal physiology, a piece of the puzzle is still missing from understanding the physiological relevance of the low-grade, prolonged systemic inflammation associated with a wide range of metabolic disorders such as obesity.

An interesting approach postulated an overlapping of the immune and metabolic systems through the nutrient-sensing pathway. Hotamisligil, in his review, explained that the metabolically triggered inflammation aims at blocking the anabolic signaling pathway (through insulin and IGF, for example) that accompanies overeating in an attempt to counteract nutrient deposition and storage (Hotamisligil, 2006). Hence, a juxtaposition between nutrients and pathogens sets the ground for chronic metabolic diseases. In support of this hypothesis, a surplus of energy is associated with hypertrophy of the adipose tissue that releases proinflammatory cytokines, including IL-6, IL-1β, and tumor necrosis factor α (TNF-α), into the circulation (Coppack, 2001). These factors activate several signaling pathways (Ott et al., 2007) and impede others, like the intracellular insulin cascade, causing insulin resistance (Ait-Lounis & Laraba-

Djebari, 2012; Mohallem & Aryal, 2020; Plomgaard et al., 2005). In contrast, knocking out TNF-α receptors 1 and 2 improved insulin sensitivity in mice consuming diet-induced obesity (Uysal et al., 1997).

The overlap between the metabolic and immune systems feeds into the explanation of low-grade inflammation in overnutrition but fails to clarify its presence among undernourished populations.

A mouse model of moderate acute malnutrition (MAM) was developed to study the interplay between undernutrition and systemic inflammation (Patterson et al., 2022). This study showed that MAM presents an inflammatory profile even in the absence of infection, exacerbating response to inflammatory triggers. These findings were mediated by intestinal dysbiosis, compromised intestinal barrier integrity, increased intestinal permeability, and systemic exposure to bacterial lipopolysaccharides (LPS) (Patterson et al., 2022). Indeed, an observational cross-section study on malnourished children affirmed the contribution of LPS in the pathophysiology of MAM (Patterson et al., 2021). Once in circulation, LPS stimulates toll-like receptor 4 (TLR4) and mediates an inflammatory response (Lu et al., 2008; Pålsson-McDermott & O'Neill, 2004). However, it is still unclear whether the increase in LPS is a cause or a consequence of malnutrition.

This particular mechanism was shown not to be exclusive to malnutrition. In fact, changes in gut microbiota have been described in the etiology of low-grade inflammation of obesity and diabetes in the context of obesogenic diets (Cani et al., 2007; Cani et al., 2008).

Whether in over or underfeeding conditions, a distinctive feature of low-grade systemic inflammation includes some hallmarks, such as a decrease in serum albumin and an increase in CRP levels.

2. Hallmarks of inflammation

a. Albumin

Albumin is the most abundant protein in circulation and accounts for 50% of total proteins (Caraceni et al., 2013). A clear-cut inverse association was described between plasma albumin concentration and mortality in population studies (Eckart et al., 2019; Fulks et al., 2010; Kunutsor et al., 2018), which has given albumin the characteristic to predict health and nutritional statuses (Bharadwaj et al., 2016).

Albumin is a major player in health and disease as it is involved in major body functions. It is responsible for free radical scavenging, thus its antioxidant property (Levitt & Levitt, 2016), capillary permeability, cell signaling processes, and many others; however, the primary function of this plasma protein is to maintain the oncotic pressure and control body compartments' fluids distribution (Moman & Varacallo, 2018). Moreover, albumin is a transport vehicle of cholesterol, bile pigments, nitric oxide, metals, and free fatty acids (FFA) (Evans, 2002). Albumin availability for binding does not take the attention it deserves from health experts. Clear evidence states that loss or abnormal function of fatty acid-binding proteins increases FFA availability and ectopic fat accumulation (Atshaves et al., 2010). In line, an in vitro experiment showed that the decrease in the concentration of bovine serum albumin in the presence of palmitate increases apoptosis and cell death (Oliveira et al., 2015). This study suggests carefully watching the ratio of FFA to albumin that would determine the

concentration of unbound FFA (Oliveira et al., 2015), known to be physiologically active (Huber & Kleinfeld, 2017).

Serum albumin consists of 585 amino acids (He & Carter, 1992) and has a half-life of 3 weeks in humans (Liumbruno et al., 2009), around 1.9 days in rats, and a single day in mice (Feng et al., 2018; Nguyen et al., 2006; Reed & Peters Jr, 1984). Table 2 shows the amino acid composition of human albumin. Interestingly, lysine ranked first as the most abundant essential amino acid in the sequence (Denko et al., 1970). Therefore, failure to provide adequate amounts of lysine is expected to affect albumin production.

Albumin production in the liver is stimulated by high-protein and high-energy diets, insulin, and growth hormones. On the contrary, it is inhibited by low-protein diets and inflammatory markers such as IL-6 and TNF-α (Caraceni et al., 2013; Chien et al., 2017). Serum albumin is a negative acute-phase protein as its concentration is reduced in case of inflammation (Chien et al., 2017). It is then predictable to see a drop in blood albumin concentration among patients with elevated serum inflammatory markers, including CRP (Eckart et al., 2019; Levitt & Levitt, 2016).

b. CRP

CRP is a non-specific marker of inflammation that is increased with the severity of the health problem. This protein is primarily produced in the liver but can also be synthesized by macrophages, lymphocytes, adipocytes, and others (Sproston & Ashworth, 2018). An increase in IL-6 and IL-1β stimulates CRP production, and TNF-α further enhances its biosynthesis (Zhang et al., 1996). CRP also contributes to

the activation of the C1q molecule, production of monocyte chemoattractant protein-1 (MCP-1), and binding to Fc receptors, resulting in the release of several pro-inflammatory cytokines like IL-8 (Sproston & Ashworth, 2018). The monomeric CRP holds 206 amino acids (Salazar et al., 2014) and has a half-life of 19 hours in humans (Vigushin et al., 1993) and 4 hours in mice (Janciauskiene et al., 2011). Table 2 presents the amino acid composition of CRP and shows that lysine is a significant contributor to its chain.

Some observations (secondary data in most cases) have acknowledged an inverse relationship between albumin and CRP levels in particular cases. Mosli & Mosli showed an inverse association between albuminemia and obesity (Mosli & Mosli, 2017), which independently elevates CRP (Bastard et al., 1999; Pannacciulli et al., 2001; Rawson et al., 2003; Visser et al., 1999). Patients with celiac disease also present alterations in their blood parameters, characterized by a drop in the levels of albumin (Brar et al., 2006) and an increase in CRP (Tetzlaff et al., 2017). Nonetheless, some researchers suggested a CRP (or hsCRP) to albumin or prealbumin ratio to identify the activity of some inflammatory diseases like Crohn's (Qin et al., 2016), ulcerative colitis (Sayar et al., 2018), cardiovascular disease (Wang et al., 2019), and even risks of mortality (Llop-Talaveron et al., 2018). Remarkably, a recent study has highlighted the use of this ratio as a marker in patients with Schizophrenia (Balcioglu & Kirlioglu, 2020).

3. LPS

LPS, an endotoxin, are composed of a lipid domain (Lipid A), an oligosaccharide chain (O-antigen), and a polysaccharide core. They tag gram-negative bacteria as they constitute their outer membrane to preserve their integrity (Farhana & Khan, 2021). LPS has gained considerable research focus and has been an issue of concern because they generate a robust pro-inflammatory stimulus in the body.

LPS can reach systemic circulation through infection or internal release by the gut microbiota; in both cases, they were proven to pose a health risk. Endotoxins find their way to the circulation either through disrupted intestinal barriers (known as leaky barriers), permitting a paracellular translocation of LPS (Ghosh et al., 2020), or through the lipid raft- and CD36-mediated transcellular uptake from the intestine into the portal vein (Akiba et al., 2020). Once in the circulation, LPS interact with the TLR4 and stimulate an inflammatory response through myeloid differentiation primary response 88 (MyD88)-dependent and independent pathways (Lu et al., 2008; Pålsson-McDermott & O'Neill, 2004), leading to the release of cytokines such as IL-6, IL-1β, and TNF-α and other proinflammatory markers (Akiba et al., 2020).

Even a small increase in plasma concentration of LPS, also known as metabolic endotoxemia, has been proven to be involved in low-grade inflammation, hence numerous diseases, such as obesity, diabetes, atherosclerosis, and non-alcoholic fatty liver disease (NAFLD), etc. (Mohammad & Thiemermann, 2021). For instance, in obesity, microbiota profiles differ (Ley et al., 2006) and circulating LPS levels rise (Trøseid et al., 2013). In addition, TLR4 mRNA and protein expressions in adipose tissues (Creely et al., 2007) and skeletal muscle of people with obesity and insulin resistance/type 2 diabetes (Frisard et al., 2010; Reyna et al., 2008) were shown to

increase compared to healthy subjects, showing an active LPS/TLR4 signaling pathway. In line, a study on children with NAFLD supports the correlation between serum concentration of endotoxin and disease activity score ≥5 (Alisi et al., 2010), suggesting the contribution of LPS to the progression of the disease.

E. Hypothesis

When a diet depends heavily on cereals, especially wheat, lysine, the amino acid that is mainly needed for the production of albumin and CRP, will be lacking in sufficient amounts. This displays an imbalance between the availability of essential amino acids for blood proteins. We hypothesize that the lack of a sufficient quantity of dietary lysine in rats mediates low-grade inflammation in the absence of infectious stimuli; and that the inflammatory profile in LPS-challenged rats would be altered in the case of lysine deficiency. Table 2 clearly shows the amino acid compositions of our proteins of interest in this work, gluten, albumin, and CRP.

Table 2. Amino acid composition of selected proteins

Amino acids	g/100g of Gluten[¥]	g/100g of Albumin[#]	g/100g of CRP[§]
Essential amino acids			
Histidine	2.5	4.8	1
Isoleucine	3.5	2	5
Leucine	6.9	11.3	7
Lysine	**2.9**	**12.4**	**7**
Methionine	1.2	1.3	1
Phenylalanine	4.7	7.4	8
Threonine	2.9	4.7	6
Tryptophan	1.1	NA	4
Valine	4.3	5.3	8
Non-essential amino acids			
Alanine	3.6	4.6	3
Arginine	4.5	6.2	4
Asparagine	NA	NA	4
Aspartic acid	5.0	10.2	4
Cysteine	2.5	NA	2
Glutamic acid	33.1	16.3	8
Glutamine	NA	NA	4
Glycine	4.1	2.4	4
Proline	10.9	3.2	5
Serine	4.6	3.2	8
Tyrosine	1.7	4.7	5
Total	100	100	100

NA is "Not Available" and means that the information is lacking in the reference.

[¥] Information about amino acid composition of gluten is retrieved and adapted from Dyets Inc. inspection data sheet.

[#]Albumin data are adapted from Denko *et al.*, 1970 (Denko et al., 1970).

[§]CRP data are adapted from Oliveira *et al.*, 1979 (Oliveira et al., 1979).

CHAPTER II

AIMS

The tight relationship between lysine and health outcomes displays a potential role for lysine deficiency in the etiology of low SES-related diseases. As previously elaborated, low SES communities rely on cereals that lack lysine in a considerable amount. We hypothesize that the lack of a sufficient amount of lysine in a diet mediates low-grade inflammation seen among these communities and contributes to the onset of NCDs. To our knowledge, this is the first study that aims at studying the effect of dietary lysine manipulation on some inflammatory hallmarks and assessing its impact in the presence of an inflammatory insult induced by LPS in young rats. This work evaluated two different levels of dietary lysine (60% and 30% below the recommended amounts) compared to a control diet. Accordingly, below are the three specific objectives of the project that were assessed using 60% and 30% lysine deficiency in two separate experiments.

1. Determine the effect of lysine deficiency of gluten diet on anthropometric measurements, energy parameters, as well as blood and tissue markers in sham and LPS-challenged young rats.
2. Determine the effect of lysine deficiency of gluten diet on selective inflammatory markers in tissues and blood in sham and LPS-challenged young rats.
3. Determine the effect of lysine deficiency of gluten diet on anxiety-like behavior of sham and LPS-challenged young rats.

CHAPTER III

MATERIALS AND METHODS

A. Animals

The protocol of these experiments was approved by the Institutional Animal Care and Use Committee (IACUC) of the American University of Beirut (AUB); approval # 20-08-577.

Five-week-old male Sprague Dawley rats, weighing around 190 grams, were placed in a controlled temperature (22±1°C) and humidity (65%) room with a light/dark cycle of 12/12 (lights start at 7 a.m.). The required number of rats per group (n=8) was calculated using previously determined weight gain data (difference in means = 6.0 g/d and SD = 0.95 g/d) and assuming a statistical power of 80% and a significance level of 5% (Hammoud et al., 2017). One week prior to each of the experiments, the rats were housed individually in wire-bottom cages and received semisynthetic control diets to be familiarized with the texture of the diet and the environmental changes. During the experiments, rats had free access to water and their corresponding experimental diets.

B. Experimental design

Two sequential experiments were run:

Experiment 1: Investigating the effect of 60% lysine deficiency in diet on the body compartment, inflammatory profile, and behavior in young male Sprague Dawley rats, and

Experiment 2: Investigating the effect of 30% lysine deficiency in diet on the body compartment, inflammatory profile, and behavior in young male Sprague Dawley rats.

Rats were randomly divided into groups of 8 except for one group that held 4 rats only (the control group of *Experiment 2*), as shown below. Different treatments and experimental diets were given according to the group to which they were assigned. A summary of the experimental design is illustrated in Figure 2.

Experiment 1: Investigating the effect of 60% lysine deficiency in diet on the body compartment, inflammatory profile, and behavior in young male Sprague Dawley rats.

Group 1- Control: Control Diet + saline injection (n=8)

Group 2- LPS: Control Diet + LPS injection (n=8)

Group 3- Lysine Deficient 60%: 60% Lysine-Deficient Diet + saline injection (n=8)

Group 4- Lysine Deficient 60%-LPS: 60% Lysine-Deficient Diet+LPS injection (n=8)

Experiment 2: Investigating the effect of 30% lysine deficiency in diet on the body compartment, inflammatory profile, and behavior in young male Sprague Dawley rats.

Group 1- Control: Control Diet (n=4)

Group 2- Lysine Deficient 30%: 30% Lysine-Deficient Diet + saline injection (n=8)

Group 3- Lysine Deficient 30%-LPS: 30% Lysine-Deficient Diet+LPS injection (n=8)

The Control Diet contained an adequate amount of lysine, while the 60% and 30% Lysine-Deficient Diet provided 60% and 30% less lysine than the Control Diet, respectively. Cereals in nature contain around 60% less lysine than animal foods (Young & Pellett, 1994), hence the rationale for using 60% deficiency.

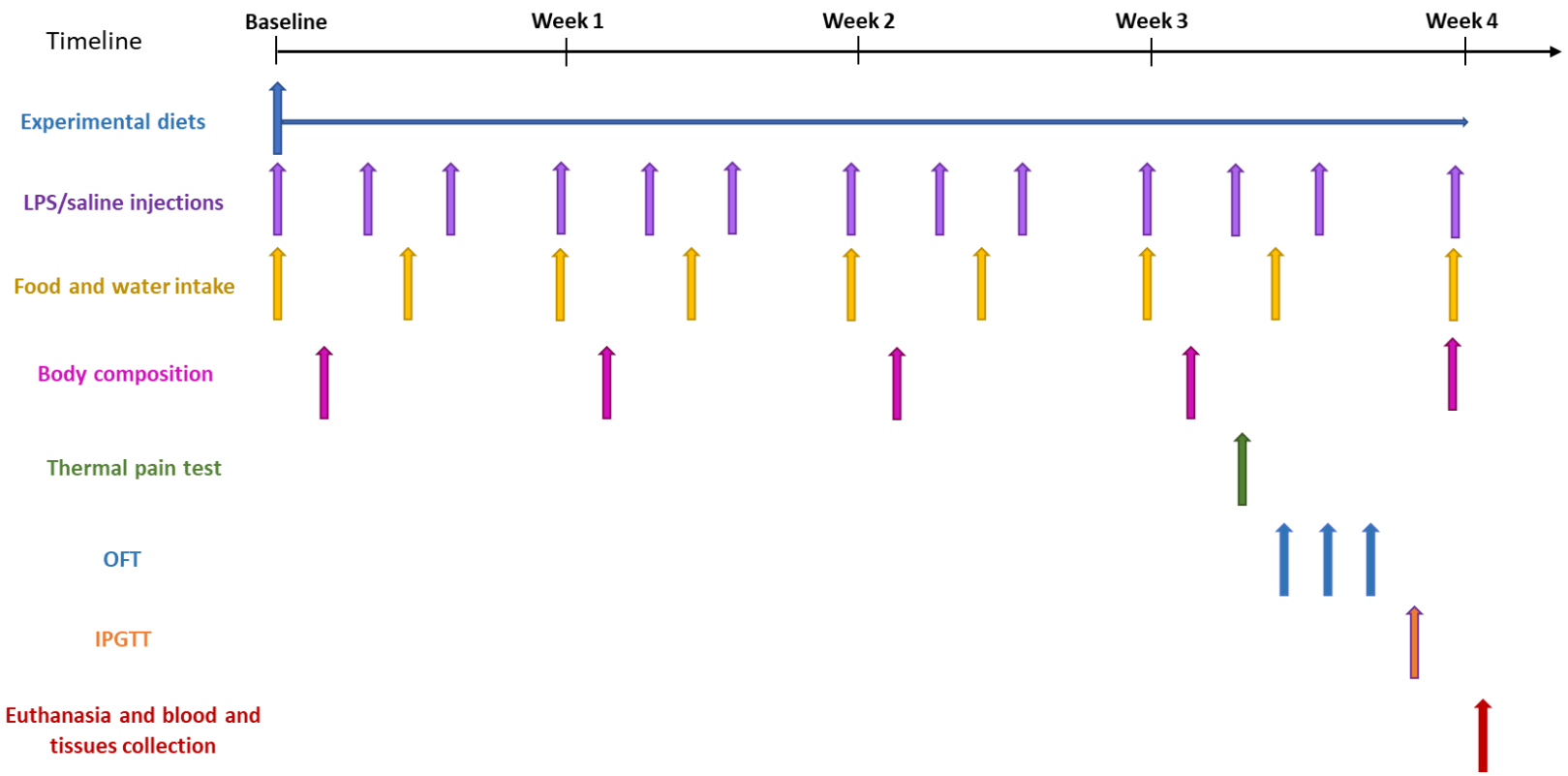

Figure 2. Summary of the experimental design

OFT: Open Field Test

IPGTT: Intraperitoneal Glucose Tolerance Test

After the adaptation period, experimental diets were given on day 0 of the experiments, at baseline. LPS/saline injections were administered 3 times per week intraperitoneally at a fixed time. Food and water intake were measured twice per week, accounting for the spillage. Body composition was performed using TD-NMR Minispec LF110 (Brucker, MAS, USA), once per week, at a fixed interval. During the last week before euthanasia, and before the LPS/saline injection time of the corresponding day, a thermal pain test, OFT, and IPGTT, were performed.

C. Diets

Three diets were prepared for this study. Ingredients were bought from Dyets Inc. (Bethlehem, PA, USA) and local stores, and diets were accustomed to fit the needs of the experiments. The different diets' compositions and nutritional facts are presented in Tables 3 and 4, respectively.

Gluten-based diets were chosen for this experiment since gluten (the wheat protein) is naturally deficient in lysine. The control diet is supplemented to meet the lysine requirements in growing rats, according to the National Research Council (NRC, 1995). The diets were designed to be isoenergetic (~3.92 kcal/g) and isonitrogenous (~15.4% of energy from protein).

Table 3. Diet composition of the experimental diets

Ingredients (g/kg)	Control Diet	60% Lysine-Deficient Diet	30% Lysine-Deficient Diet
Wheat gluten*[ǀ]	188.2	196.1	192
Cornstarch	388.1	387.6	388.1
Sucrose	232	232.1	232
Corn oil	70	70	70
Cellulose*	50	50	50
Mineral mix*[€]	35	35	35
Vitamin mix*[£]	10	10	10
Choline Bitartrate	2.5	2.5	2.5
L-Methionine	3	3	3
L-Threonine	0.5	0.5	0.5
Potassium phosphate*[¥]	13.2	13.2	13.2
L-Lysine HCl*[¶]	7.5	-	3.7

*Obtained from Dyets Inc., Bethlehem, Pennsylvania, USA.

[ǀ]According to the company's composition sheet, wheat gluten contained 76% of protein.

[€]"Phosphorus-free" mineral mix (AIN-93G mineral mix phosphorus-free, used as 35 g/kg of diet).

[£]Vitamin mix (AIN-93VX vitamin mix, used as 10 g/kg of diet).

[¥]Potassium phosphate KH2PO4, molecular weight 136 g/mol, of which phosphorus 31 g/mol.

[¶]L-Lysine HCl, molecular weight 182 g/mol, of which lysine 146 g/mol.

Table 4. Nutrition information of the experimental diets

Nutrients	Control Diet	60% Lysine-Deficient Diet	30% Lysine-Deficient Diet
Protein* (%)	15.4	15.4	15.4
Carbohydrates* (%)	67.9	67.9	67.9
Fat* (%)	15.9	15.9	15.9
Total energy (kcal/kg)	3924	3926	3925
Lysine (g/kg)	9.8	3.8	6.8

* values are presented as a percentage of total energy

The nutrient content of diets was estimated based on the nutrition information on the label of the products.

D. Intraperitoneal LPS administration protocol

An LPS challenge was added to the protocol to investigate whether the presence of lysine (in control diets) can modify the inflammatory response and whether lysine deficiency (in different levels) alters the existing inflammation. Salmonella Typhosa LPS (Sigma Aldrich, St. Louis, MO, USA) was solubilized in 0.9% saline solution for a final concentration of 20 μg/ml and administered intraperitoneally at a dose of 50 μg/kg, three times per week which sums up to a total quantity of 150 μg/kg per week. This dose was expected to affect the rats' food intake, body composition, inflammatory status, and behavior, while the frequency would be in line with the 1.9 day of the half-life of the plasma albumin in rats (Reed & Peters, 1984). Unchallenged groups received a similar volume-to-weight intraperitoneal (IP) injection of 0.9% saline solution.

LPS is an endotoxin derived from Gram-negative bacteria. It was used since it is known to induce an immune reaction, activate liver macrophages, and stimulate the production of inflammatory cytokines (Liu et al., 2017).

The literature does not provide consistent data about the recommended dose of LPS per kilogram of body weight to induce a low-grade level of systemic

inflammation. A study by Bison et al. tested the effect of different doses of LPS (1, 5, 15, 50, 125, or 250 μg/kg) in adult male Sprague Dawley rats and conveyed that even a single injection of the lowest dose was able to affect TNF-α, while changes in interleukins were witnessed at 5 μg/kg (Bison et al., 2009). Nevertheless, changes in body weight and behavior were seen 24 hours post-injection at a dose of 50 μg/kg. Supporting data by Elgarf et al. showed that repeated exposure (6 injections every other day) to 50 μg/kg over two weeks was the lowest effective dose that significantly produced behavioral changes using a forced swimming test and social interaction test in rats (Elgarf et al., 2014).

E. Food and water intake

Food and water intakes were measured twice per week during the experimental period. Measurements were determined to the nearest 0.1 g by weighing feed containers and water bottles before and after adjustments. Feed spillage was collected and weighed, and food intake was corrected for spillage. Energy intake was then calculated by multiplying the weight of food consumed by 3.92 kcal/g.

F. Body weight and composition

Rats were weighed and their body composition was assessed once per week using TD-NMR Minispec LF110 (Brucker, MAS, USA). The device was set and calibrated before use. Rats were placed in a plastic cylinder and kept immobile for about 2 minutes, the duration needed for the completion of the scan. This device provides a noninvasive body compartment analysis in living animals based on a magnetic resonance system. Lean body mass (LBM), fat mass (FM), and extracellular fluid (ECF)

were the main outcomes; changes from baseline and across the experimental period were calculated.

G. Energy balance parameters

Total Energy Expenditure (TEE_{xp}) was estimated based on the equation by Ravussin *et al.* that depends on energy intake (EI) and accumulated energy stores in tissues, represented by FM and LBM over the whole experimental period (Ravussin et al., 2013):

TEE_{xp} (kcal) = EI (kcal) – (FM_{stores} (kcal) + LBM_{stores} (kcal))

where $FM_{stores} = \Delta FM_{gain} = (FM_{final}$ (g) – $FM_{initial}$ (g)) x 9.4 (kcal/g)

and, $LBM_{stores} = \Delta LBM_{gain} = (LBM_{final}$ (g) – $LBM_{initial}$ (g)) x 1.0 (kcal/g)

The caloric equivalence of FM and LBM were assigned as 9.4 and 1 (kcal/g), respectively.

Also, Energy Efficiency (EE) is determined as the amount of weight gained (WG) per 100 kcal consumed and was computed by dividing weight gain by the energy consumed.

EE = (WG (g) x 100 kcal)) ÷ EI (kcal)

H. Intraperitoneal glucose tolerance test (IPGTT)

Room temperature, pH-adjusted 50% glucose solution (Sigma-Aldrich, MO, USA) in 0.9% saline was administered intraperitoneally to 4 rats per group at a volume that corresponds to 2 g/kg after a 5-hour-fast. A tail prick with a small needle was done to collect blood drops from the lateral tail vein. The glycemic response was measured with Accu-Check® Guide (Mannheim, Germany) over two hours at

different time points (baseline before IP injections and 15, 30, 90 and 120 min after injections). The area under the curve (AUC) was calculated using Prism, GraphPad software (CA, USA).

I. Blood and tissue collection

By the end of the fourth week, the rats were euthanized by severing their hearts after a 5 to 6-hour fast, twenty-four hours after the last LPS/saline injection. The procedure was performed under isoflurane (Forane®; Abbott, Berks, UK). Blood was collected in EDTA tubes from the superior vena cava, and blood samples were centrifuged at 3,500 RPM for 15 minutes at 4ºC (Eppendorf 5810R, Hamburg, Germany). The liver, spleen, and abdominal epididymal fat were immediately excised, weighed, snap-frozen in liquid nitrogen, and then stored at -80ºC.

J. Fasting plasma analysis

Plasma glucose (Glu), total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, triglycerides (TG), albumin (Alb), lactate, chloride, and sodium were determined using Vitros® 350 Chemistry System (Ortho-Clinical Diagnostics, NJ, USA). Low-density lipoprotein (LDL) cholesterol was calculated using Friedewald equation: LDL = TC – HDL – TG/5 (Friedewald et al., 1972).

Plasma insulin (Ins) (Merck Millipore, USA), total glucagon-like peptide-1 (GLP-1) (Merck Millipore, USA), and vasopressin (Elabscience, USA) were measured via enzyme-linked immunosorbent assay (ELISA) kits and quantified using Thermo Scientific Multiskan FC microplate reader (NY, US). HOMA-IR index was computed

using the equation in HOMA-IR = Ins (mmol/L) x (Glu (mmol/L)) ÷ 22.5 (Antunes et al., 2016).

K. Hepatic fat extraction

Frozen liver sections (around 3 g) were freeze-dried (Benchtop Freeze-Dry System, Labconco, MO, USA) for 48 hours for an expected water loss between 65 and 70%. Samples were then put in moisture-free sealable filter bags, and hepatic fat content was determined using an ANKOM^{XT10} extractor (ANKOM Technology 2052 O'Neil Road, NY, USA) with petroleum ether as a solvent (BP 40-60ºC) for a cycle of 40 minutes. After extraction and cooling, samples were put for 30 min at 100ºC to dry and then cooled at room temperature in a desiccator. The difference in weight refers to the extracted fat from liver samples, and the fat percentage was then inferred.

L. Amino acid analysis

Amino acid determination is based on the high-performance liquid chromatography (HPLC) method done by Mann et al. (Mann et al., 1988). Plasma samples (20 µL) were mixed with an equal volume of an internal standard (L-homoserine, Sigma Aldrich, MO, USA) and HPLC-grade water for a final volume of 200 µL. Then, after protein precipitation with perchloric acid, the samples were vortexed, centrifuged at 15,000×g for 10 minutes at 4ºC, filtered, and 10 µL of the filtered sample was injected into the HPLC system. The derivatized amino acids were separated by o-phthalaldehyde on a Hichrom Spherisorb ODS reverse phase column (150 × 4.6 mm; Hichrom Ltd. Berks, UK) at a flow rate was 1.2 ml/minute. The

retention times of the amino acids are presented in Table 5. Twenty amino acids were identified and quantified by dividing the area under the curve for each amino acid by the area of the internal standard.

Table 5. Retention time of amino acids separated by HPLC

Amino acids	Retention Time (minutes)	Amino acids	Retention Time (minutes)
Glutamic	10.0±0.3	Taurine	22.8±0.4
Asparagine	14.0±0.3	Tyrosine	26.0±0.5
Serine	14.3±0.3	Valine	30.0±0.5
Glutamine	16.7±0.4	Methionine	31.2±0.5
Glycine	17.7±0.4	Isoleucine	34.8±0.5
Threonine	18.1±0.4	Tryptophan	35.2±0.6
Histidine	18.9±0.4	Phenylalanine	35.6±0.5
Citrulline	19.8±0.4	Leucine	36.5±0.5
Alanine	21.4±0.4	Ornithine	37.4±0.6
Arginine	22.3±0.4	Lysine	39.2±0.6

Data are expressed as the mean±SD of the retention times of amino acids (n=32).
Chromatograms showing retention times are displayed in Figures 18 and 40.

M. Histological analysis

Frozen livers and epididymal fat pads were sectioned, embedded in cassettes, and fixed in 10% buffered formalin solution over 24 hours. Then, tissues underwent dehydration by sequential incubation in different percentages of ethanol from 70%, 90%, 95%, and 100% over 24 hours before being placed in stainless-steel molds for paraffin embedding. Paraffin blocks were then placed in a Microtome, and thin sections were cut and mounted on microscope slides. After complete dryness, the slides underwent deparaffinization and rehydration to distilled water before staining with Hematoxylin and Eosin (H&E).

Slide images were captured with Olympus CX41 microscope (Olympus Corp, Tokyo, Japan). Areas of adipocytes were measured using ImageJ software (MD, USA), and quantifications were performed manually for a set area in all slides. A pathologist blinded to the experimental groups evaluated the morphology of liver sections.

N. Gel electrophoresis and Western blot analysis

Liver and epididymal fat pad were separately homogenized with radio-immunoprecipitation assay (RIPA) lysis buffer containing a cocktail of phosphatase and protease inhibitors (BioWorld, OH, USA). Homogenates were centrifuged at 13,600×g for 20 minutes at 4°C, and protein lysates were quantified using the DC Protein Assay by Bio-Rad (CA, USA) based on the Lowry assay according to the manufacturer's protocol. Equal amounts of protein lysates (around 30 µg) were diluted in 4x Laemmli loading buffer (Bio-Rad, CA, USA), heated at 95°C for 5 min, and separated by gel electrophoresis (12% SDS-PAGE for 2 hours at 90V). Gels were then transferred onto 0.45 µm nitrocellulose membrane using the semi-dry transfer method (Bio-Rad, CA, USA). Membranes were blocked with 1% gelatin in tris-buffered saline (TBS) with 0.1%Tween 0.1% for 1 hour at 37°C and then incubated overnight at 4°C with one of the following primary antibodies: polyclonal rabbit anti-β-actin (1:100, Bio-Rad) and polyclonal rabbit anti-CRP (1:1000, MyBioSource). Next, and after extensive washing, membranes were incubated at room temperature for 1 hour with an IgG HRP-conjugated secondary goat anti-rabbit antibody (1:40,000, Bio-Rad). Immunoreactive bands were visualized with enhanced chemiluminescence detection solutions (Biorad, Clarity™ Western ECL Blotting Substrates) using a ChemiDoc MP Imaging System (Bio-Rad, CA, USA). The

optical density of the bands was quantified with ImageJ (MD, USA) and normalized over the corresponding loading reference band (β-actin).

O. RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

1. RNA extraction

a. Liver

Total RNA was extracted from frozen tissue with Trizol (TRI Reagent® (Sigma-Aldrich, MO, USA). Briefly, around 30 mg of liver was homogenized with 1 ml of Trizol in Bead Ruptor Elite (Omni, International Inc., Conn, USA) for one cycle of 15 s for a speed of 4 m/s. After, 0.2 ml of chloroform was added to the mix, vortexed for 1 minute, incubated for 15 minutes, and then centrifuged at 12,000×g for 20 minutes at 4°C. After centrifugation, three different layers appear, of which the top, colorless aqueous upper phase contains the RNA. Around 400 µl of this upper layer was aspirated and transferred into a new tube. Isopropanol was subsequently added (300 µl), followed by a new cycle of centrifugation at 15,000×g for 20 minutes at 4°C to precipitate the RNA. An RNA pellet was formed at the bottom of the tube, and the supernatant was decanted. The pellets were then washed with 75% ethanol (0.5 ml) and centrifuged at 12,000×g for 20 minutes at 4°C. RNA pellets were air-dried for 10 minutes, and resuspension was done with RNAse-free water by repeated pipetting with a micropipette.

b. Fat

Special consideration was given to the adipose tissue while following the same previously described procedure. Around 150 mg of the epididymal fat pad was homogenized in Trizol. After homogenization, samples underwent centrifugation at 12,000×g for 10 minutes at 4ºC, and the upper-fat layer was removed and discarded before adding the chloroform.

2. RNA quality and quantity check

After extraction and before moving to the reverse transcription stage, RNAs were assessed using two different methods. The concentration and purity of the RNAs were verified using the DeNovix DS-11 Series Spectrophotometer (DE, USA). Absorbance was measured at 230 nm, 260 nm, and 280 nm, and ratios were computed. Accepted purity values are presented in Table 6 below. Samples that did not meet the quality criteria had their RNA extraction repeated. The target concentration was between 400 and 800 ng/µl; low-concentration samples were discarded, and high-concentration samples were diluted with nuclease-free water.

Table 6. RNA purity criteria

Absorbance	**Low**	**Ideal**	**High**
260/280	<1.9 Acidic contamination like phenols and proteins	~ 2.0	>2.2 Basic contamination
260/230	<1.8 Carbohydrate carryover, Residual phenol from nucleic acid extraction, etc.	~ 2.0	>2.2

Information retrieved from Thermo Scientific Technical Bulletin for NanoDrop Spectrophotometers (ThermoScientific, 2013).

RNA quality was further confirmed via electrophoresis. RNA samples were diluted in nuclease-free water, mixed with 6x DNA loading dye, and separated on 1% agarose gel with ethidium bromide for 50 minutes at 140V. Reading was done on ChemiDoc MP Imaging System (Bio-Rad, CA, USA). Images were subjectively evaluated. RNA samples showing two clear, well-defined bands representing the 28 and 18 subunits of the ribosomal RNA (Figure 3A) are considered "good-to-go" samples, while the smears on the gel (Figure 3B) indicated that the RNAs were degraded; thus, samples were discarded, and extractions were repeated.

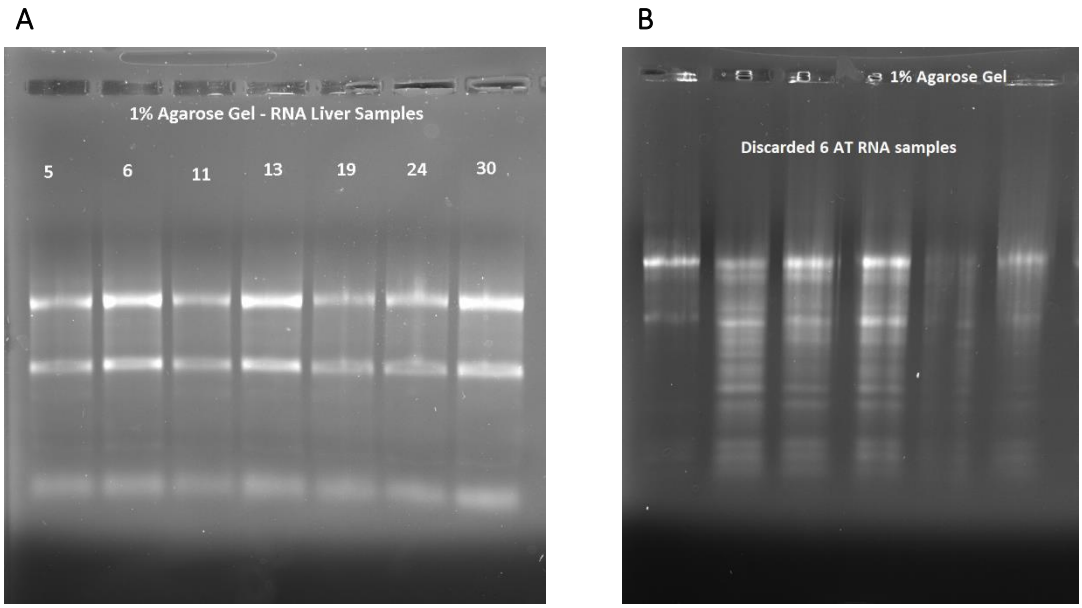

Figure 3. Sample images of RNA quality
RNA samples were separated on agarose gel stained with ethidium bromide. Figure A shows successful RNA samples, while Figure B indicates that RNAs were degraded.

3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The reverse transcription was performed using RT Master Mic for qPCR II kit (gDNA digester plus) according to the manufacturer's recommendation (MedChem Express, NJ, USA). Briefly, 3 µl of the 5× Super RT Mix was added to 2 µg of RNA

(based on the quantification) and incubated for 2 minutes at 42ºC. Next, cDNAs were transcribed after adding 5 µl of the 4× Super RT Mix to the mix that underwent a cycle of 5 minutes at 25ºC, 1 hour at 55ºC, and finally 2 minutes at 85ºC.

cDNA samples were diluted 1:3 in nuclease-free water and stored at -20ºC pending qRT-PCR runs. The mRNA expression was analyzed using qRT-PCR (Bio-Rad CFXTM Manager Software) in SYBR green master mix (Applied Biosystems, MA, USA). The list of primers used, their sequences, and their details are listed in the Table 7.

Table 7. Primer sequences used in qRT-PCR

Rattus norvegicus gene	Sequence (5' → 3')	Product size (pb)	Melting temperature (ºC)
β-actin	F: CCCGCGAGTACAACCTTCTTG R: GTCATCCATGGCGAACTGGTG	71	85
18S rRNA	F: AAACGGCTACCACATCCAAG R: CCTCCAATGGATCCTCGTTA	155	83
YWHAZ	F: CCCACTCCGGACACAGAATA R: TGTCATCGTATCGCTCTGCC	91	82
TLR4	F: GAGGTTGCTGTTCTTATTCTGAT R: GAGTGCTGAAAGTCCAGGTATT	133	80
MyD88	F: GAAATACATACGCAACCAGCAGAAA R: CAGATGAAGGCGTCGAAAAGC	156	86
IL6	F: ACAAGTCCGGAGAGGAGACT R: ACAGTGCATCATCGCTGTTC	167	80
IL6R	F: AGCAGGCAATGCTACCATTCAC R: GTCGGTATCGAAGCTCGAATTG	611	86
IL1β	F: AGGCTGACAGACCCCAAAAG R: GGTCGTCATCATCCCACGAG	264	85
Caspase 3	F: GGAGCTTGGAACGCGAAGAA R: ACACAAGCCCATTTCAGGGT	169	80
TNFα	F: CGTCCCTCTCATACACTGGC R: GCTTGGTGGTTTGCTACGAC	356	87
INFγ	F: TTGGTTTTGCAGCTCTGCCT R: GTTCACCTCGAACTTGGCGA	348	82

pb: base pair
Primers were brought from Macrogen (South Korea).

The following temperature protocol was used: 95ºC for 5 min (for denaturation) and 40 cycles of 95ºC for 10 seconds (s) and 57ºC for 30 s followed by 72ºC for 30 s. In all runs, reactions were performed in duplicates using two different housekeeping genes (β-actin, 18S rRNA, and/or YWHAZ), negative and positive controls. Expression levels of the target genes were calculated according to the ΔΔCt method using tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) gene as an internal control for normalization. Changes in gene expression were presented relative to the control group in each of the two experiments.

After completion of the amplification, qRT-PCR products were loaded onto 2% agarose gel stained with ethidium bromide for the identification of the amplicons and to confirm the exactitude of the readings.

P. Plantar test for thermal stimulation - Hargreaves test

Rats were put for 30 minutes in a transparent glass-floored chamber at a temperature of 32ºC for acclimatization. When rats were still and standing on the four paws, infrared heat source was projected from a 160-watt light bulb underneath the chamber to the planter surface of the rat's hind paw. This heat stimulus was applied thrice for each rat, separated by an interval of 5 minutes. The average of the two closest values was reported. The withdrawal latency, the time required to elicit a withdrawal response after heat exposure, is recorded in seconds. A cutoff latency of 18 seconds is used to avoid any tissue damage.

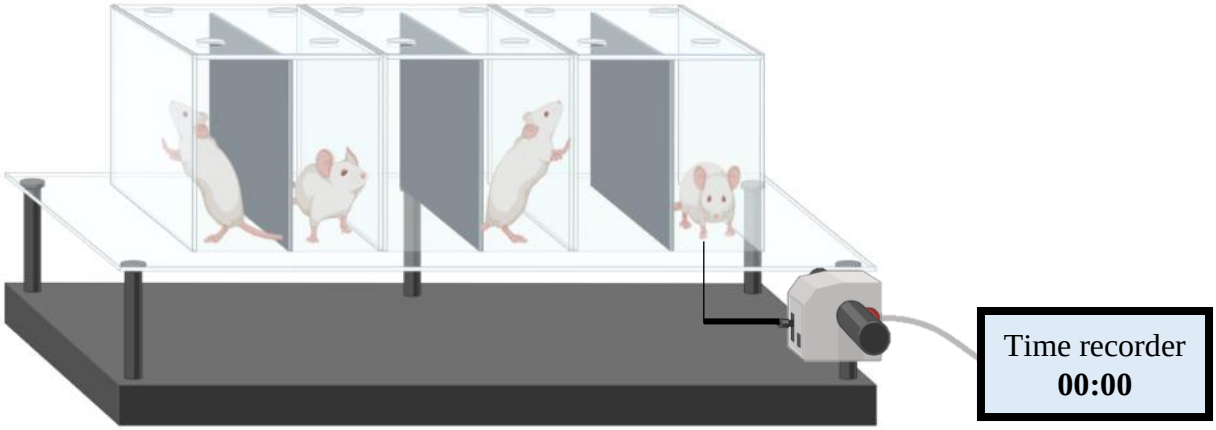

Figure 4. Illustration of the Hargreaves test
Figure showing the display and apparatus of the thermal pain test. A heat stimulus was applied thrice for each rat and average values were recorded. Image created with BioRender.

Q. Open field test and novel object recognition

An Open-Field Test (OFT) was performed on all rats to assess their locomotricity, recognition memory, anxiety-like behavior, as well as exploratory and hyperactive behaviors. This test took place during the last week before euthanasia, on three consecutive days, with an addition of a new object every day. All animals were accustomed to the experimental room for 30 minutes before the beginning of the tests.

Every rat was placed alone in an enclosure, square (W: 80 cm, L: 80 cm, H: 40 cm), open lit, and surrounded wall apparatus, enlightened in the center, where new objects were added, and dark by the periphery. Areas of the apparatus that were used in the analysis are defined in Figure 5. The rats' behaviors and trajectories were recorded for 5 minutes via a video camera connected to the tracking software Smart 3.0 (PanLab Harvard Apparatus, MA, USA). The apparatus was cleaned with 70%

ethanol between rats to ensure that no cue smell remained from the previous trial. The main outcomes of the OFT that helped identify the rats' behaviors are the total distance traveled, the distance traveled in different areas**,** and the time spent in the corners.

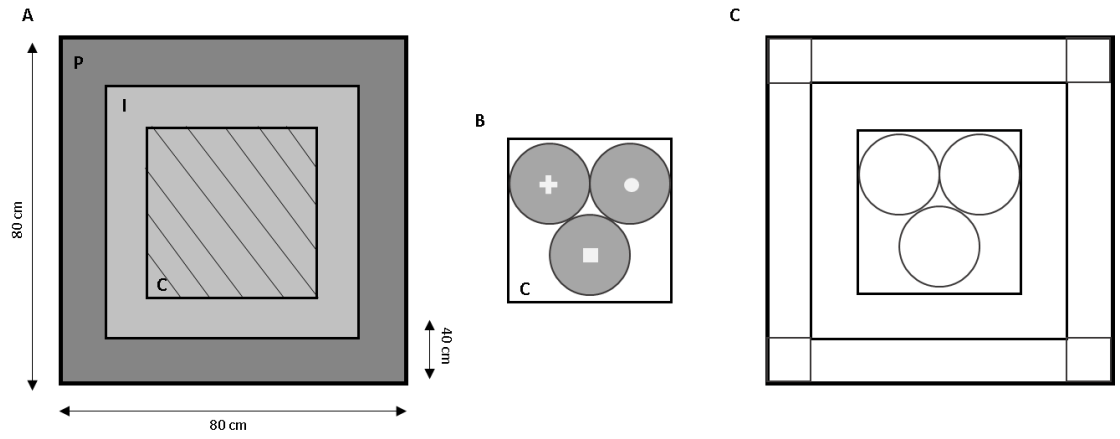

Figure 5. Schematic presentation of the open field test
(A) Definition of the Open Field Test arenas (B) Central objects definition, and (C) Scheme of the arenas used for analysis
P: the dark shadow, refers to the peripheral area (including the corners).
I: the light shadow (including C), refers to the internal area.
C: the dashed square, refers to the central area.
□ the square refers to a cube and was the first object added on day 1
✚ the cross refers to a small cross-stand base and was added on day 2
○ the circle refers to a bottle and was added on day 3

R. Statistical analysis

Data are presented as mean±standard deviation (SD) or standard error of the mean (SEM). Statistical analysis was performed using IBM Statistical Package for the Social Sciences 25 software (IBM SPSS, USA). Values were considered outliers and excluded from analysis when they surpassed the mean±2SD, except for the OFT, where all values were kept. Results were analyzed by one-way analysis of variance (ANOVA) to detect the difference among groups, followed by Fisher's least significant difference (LSD) for

posthoc multiple comparisons. General Linear Model (GLM) was also used to assess how the deficiency of lysine in the experimental diets (60% deficiency in *Experiment 1* and 30% in *Experiment 2*) (Lysine) and the administration of LPS (LPS) affected the continuous variables. A two-way repeated measures ANOVA was also used to check for the difference in the OFT between the groups and the three consecutive sessions to assess the difference in means between groups (Group) over three-time points (Session). A *p*-value less than 0.05 was considered statistically significant.

CHAPTER IV

RESULTS

The results of this work will be presented following their corresponding experiments. E*xperiment 1* discusses findings from the 60% lysine deficiency and E*xperiment 2* presents the outcomes of the 30% lysine deficiency. The sequence of the results is as follows: anthropometric measurements, intakes and energy metabolism, organs and blood parameters, albumin and CRP as parallel outcomes, inflammatory factors, and behavioral observations.

A. Experiment 1

Investigating the effect of 60% lysine deficiency in diet on the body compartment, inflammatory profile, and behavior in young male Sprague Dawley rats.

1. Body composition, food intake, and energy parameters

Body weight and composition changed differently in the presence or absence of a sufficient amount of lysine (control versus lysine-deficient diet) throughout the experimental period (Figure 6). Over 4 weeks, weight gain of the lysine-sufficient groups was about 200 g higher than that of the lysine-deficient groups. This was mainly related to the failure of lysine-deficient groups to gain weight. At the same time, weight gain of the LPS groups was slightly but significantly lower than their corresponding dietary groups. Though, this may not be of clinical relevance (Figure 6A). The changes in fat and lean mass accretions paralleled that of weight gain, and at the end of the

experimental period, differences of about 40 g of fat (Figures 6B) and 100 g of lean mass (Figures 6C) were detected in favor of groups having a control diet. Fat but not lean mass accumulation was lower among LPS-administered groups (Figures 6B and 6C). Moreover, the extracellular water was similar at baseline (*p*-value=0.645), while the discrepancies attributed to lysine deficiency increased with time to reach a *p*-value of <0.001 four weeks later (Figure 6D).

The paralleled changes in the varied components of body composition resulted in a sustained percentage of their partitioning. Indeed, fat percentages were comparable among the groups at baseline and remained similar 4 weeks later (*p*-value>0.05) (Figure 7).

Food intake was found to be comparable between the rats (*p*-value=0.249) (Figure 8A), while water intake was significantly lower among the lysine-deficient groups (*p*-value< 0.001) with a strong effect attributed to lysine deficiency (*p*-value< 0.001), but no impact seen for LPS (*p*-value=0.518) (Figure 8B).

Energy efficiency (g/100 kcal) dropped significantly with lysine deficiency, and LPS injection was able to reduce it further (Figure 9A). In contrast, energy expenditure was increased in the lysine-deficient groups, but statistical significance was only reached for the group with LPS (Figure 9B).

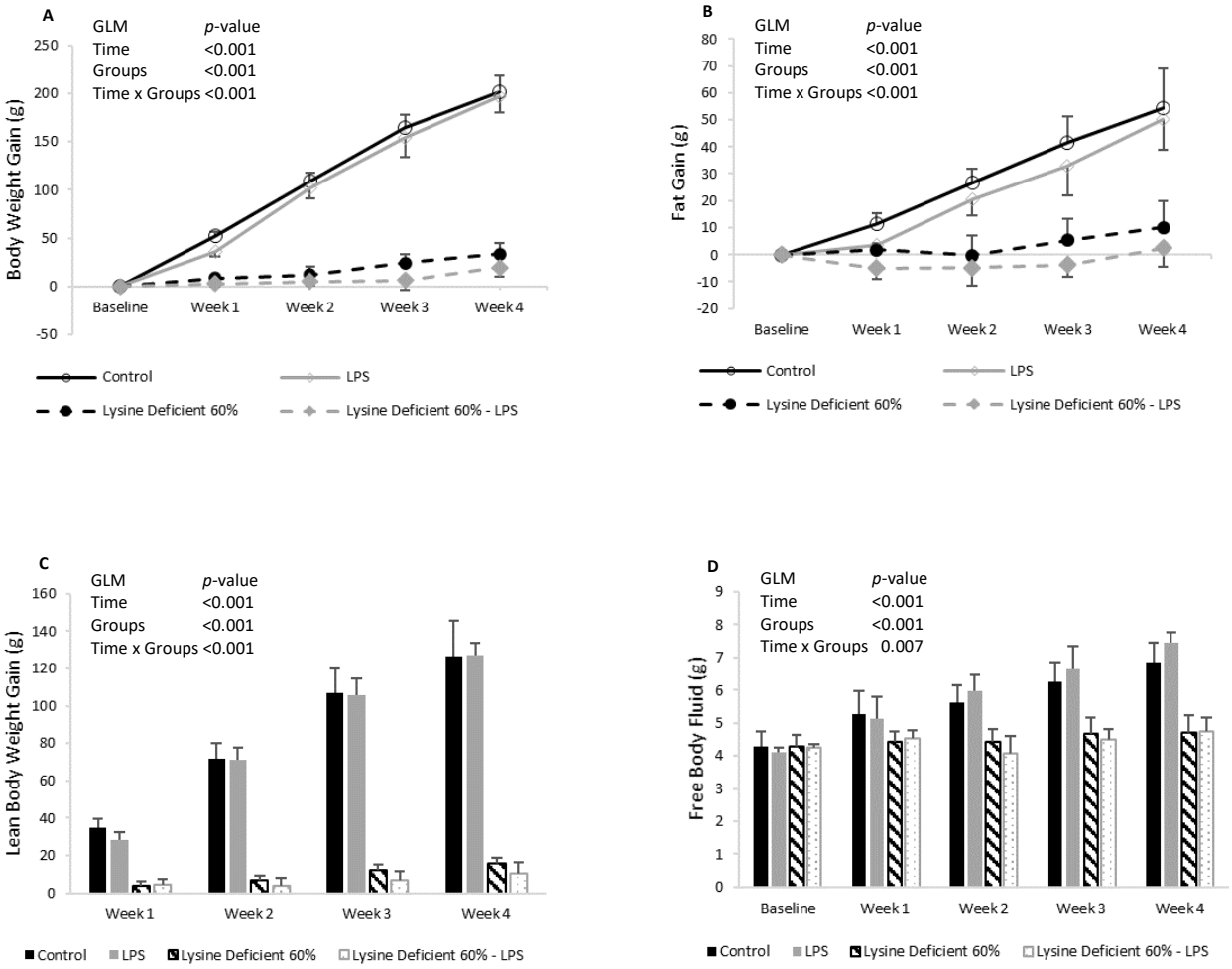

Figure 6. Body weight and composition of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Body weight gain from baseline for 4 weeks, (B) Fat gain from baseline for 4 weeks, (C) Lean body weight gain from baseline for 4 weeks, and (D) Free body fluid from baseline for 4 weeks of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group). Data are expressed as the mean±SD. General linear model (GLM) was used to analyze the effect of different parameters across the experimental period, considering the two fixed factors (Time and Groups) and their interaction (Time x Groups).

Statistical significance was set at p-value<0.05.

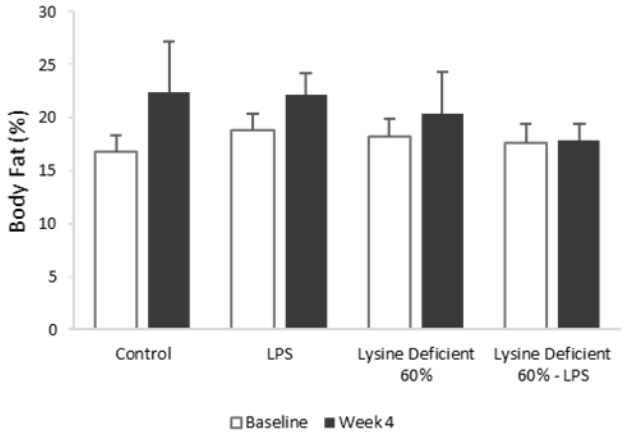

Figure 7. Percentage of body fat of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

Percentage body fat of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group) at baseline and week 4.

Data are expressed as the mean±SD. One-way ANOVA was performed to check the difference at baseline and week 4.

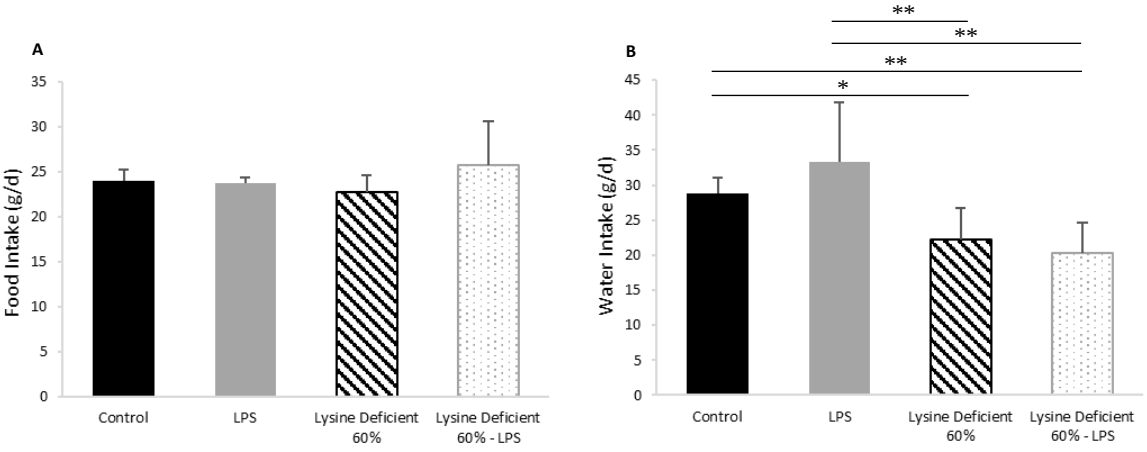

Figure 8. Food and water intake of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Food intake and (B) Water intake per day of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group).

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01).

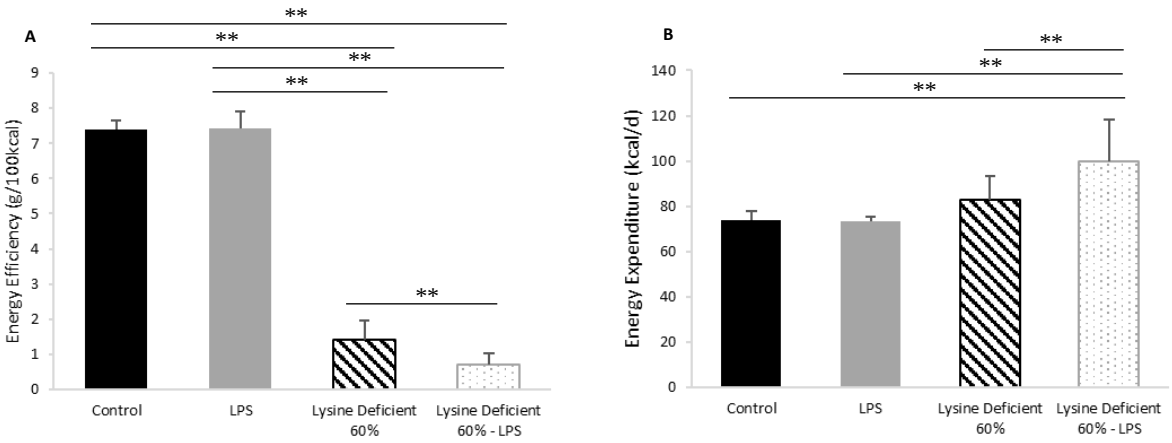

Figure 9. Energy balance of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Energy efficiency and (B) Energy expenditure per day of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group).

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (** p-value<0.01).

2. Organs

Organs were excised and weighed four weeks after the beginning of the study. Absolute weights of the organs are presented in Table 8. Organs' weights were then adjusted to the total body weights of rats on the day of euthanasia and expressed per 100 g of body weight (Figure 10). Both absolute and adjusted weights were different among groups.

The absolute spleen weight was significantly reduced with lysine deficiency and increased with LPS administration, making the weight of the LPS group the highest among all and the Lysine Deficient 60% group the lowest. However, when spleen weights were adjusted to total body weight, the difference between the Control and Lysine Deficient 60% group disappeared, while that of the LPS groups remained (*p*-value<0.001). The LPS alone induced a 50% enlargement of spleens, while interaction between lysine deficiency and LPS exhibited around a 30% increasing effect that differed from that of LPS alone.

The difference in the epididymal fat pad was independent of the body weight since the difference in the weight of the tissue remained the same after adjustment. It was shown that lysine deficiency significantly decreased fat accumulation and weight of the tissue (*p*-value<0.001). In contrast, no significant effect was observed for LPS (Figure 10B). A very strong positive association (R^2=0.9439, (*p*-value<0.001) connect epididymal fat mass to total body fat.

Interestingly, the liver exhibited a pattern that was similar to the spleen. after adjustment, liver weights were decreased with lysine deficiency (*p*-value=0.012) and increased with LPS (*p*-value<0.001) (Figure 10C). This results in a neutralizing effect of the interaction between lysine deficiency and LPS (*p*-value=0.548). It is

worth noting that the difference in liver weights was irrespective of the fat content in livers (*p*-value=0.067) (Figure 10D).

Epididymal adipose tissues were sectioned and stained with H&E. Microscopic images are displayed in Figure 12 and present apparent differences among experimental groups. After sectioning and staining, adipocytes were subjected to descriptive analysis to identify their relative numbers and sizes. The number of adipocytes increased with both lysine deficiency (*p*-value<0.001) and LPS (*p*-value=0.023). An opposite pattern was seen in adipocyte size; it was significantly smaller in both lysine-deficient groups, attributing the effect on the size to lysine deficiency (*p*-value=0.001) but not LPS (Figure 13).

Liver histology was assessed, and no morphological changes were described. Mononuclear cell infiltration was detected neither at lobules nor at the portal tracts levels (Figure 14).

Table 8. Weight of the organs of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

Organs (g)	Control	LPS	Lysine Deficient 60%	Lysine Deficient 60%-LPS	*ANOVA* *p*-value
Spleen	0.82±0.09[a]	1.27±0.09[b]	0.51±0.07[c]	0.63±0.05[d]	**<0.001**
Epididymal Fat Pad	4.76±0.97[a]	4.55±0.70[a]	2.07±0.51[b]	1.73±0.36[b]	**<0.001**
Liver	16.27±1.46[a]	17.72±1.48[b]	8.96±1.13[c]	8.85±0.91[c]	**<0.001**
Final Body Weight	388.2±19.9[a]	384.2±17.6[a]	220.9±16.0[b]	206.5±17.2[b]	**<0.001**

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons (n=8 per group). Means with the same letter are not significantly different (p-value>0.05).
Statistical significance was set at p-value<0.05.

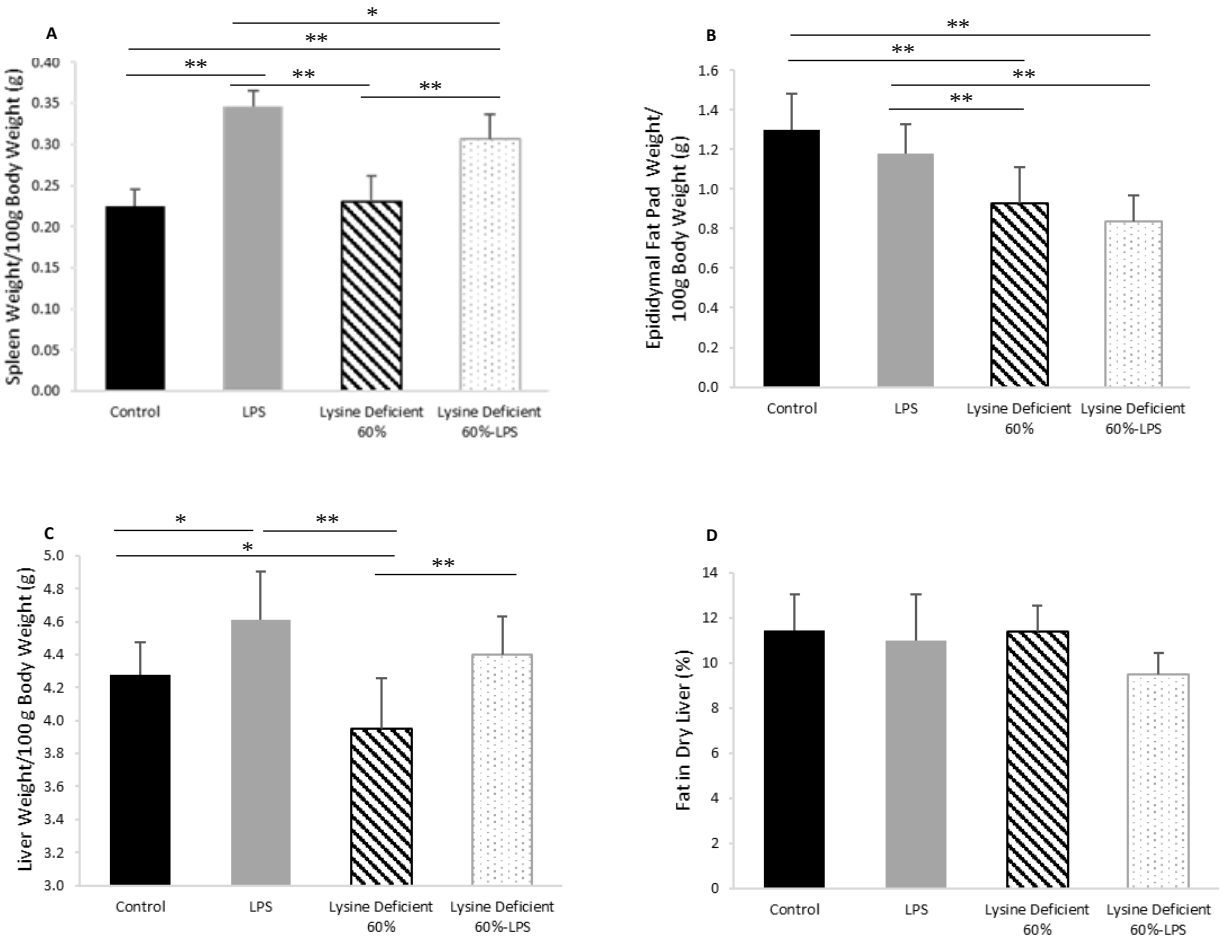

Figure 10. Adjusted organs weights and liver fat content of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Spleen, (B) Epididymal fat pad, and (C) Liver weights per 100 g of body weight, and (D) Percentage of fat content in dry livers of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group).

Data are presented as mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01).

Figure 11. Correlation between epididymal fat pad and total body fat
A strong positive liner relationship exists between body fat assessed by week 4, a day before euthanasia and edidymal fat pad weighed after euthanasia.

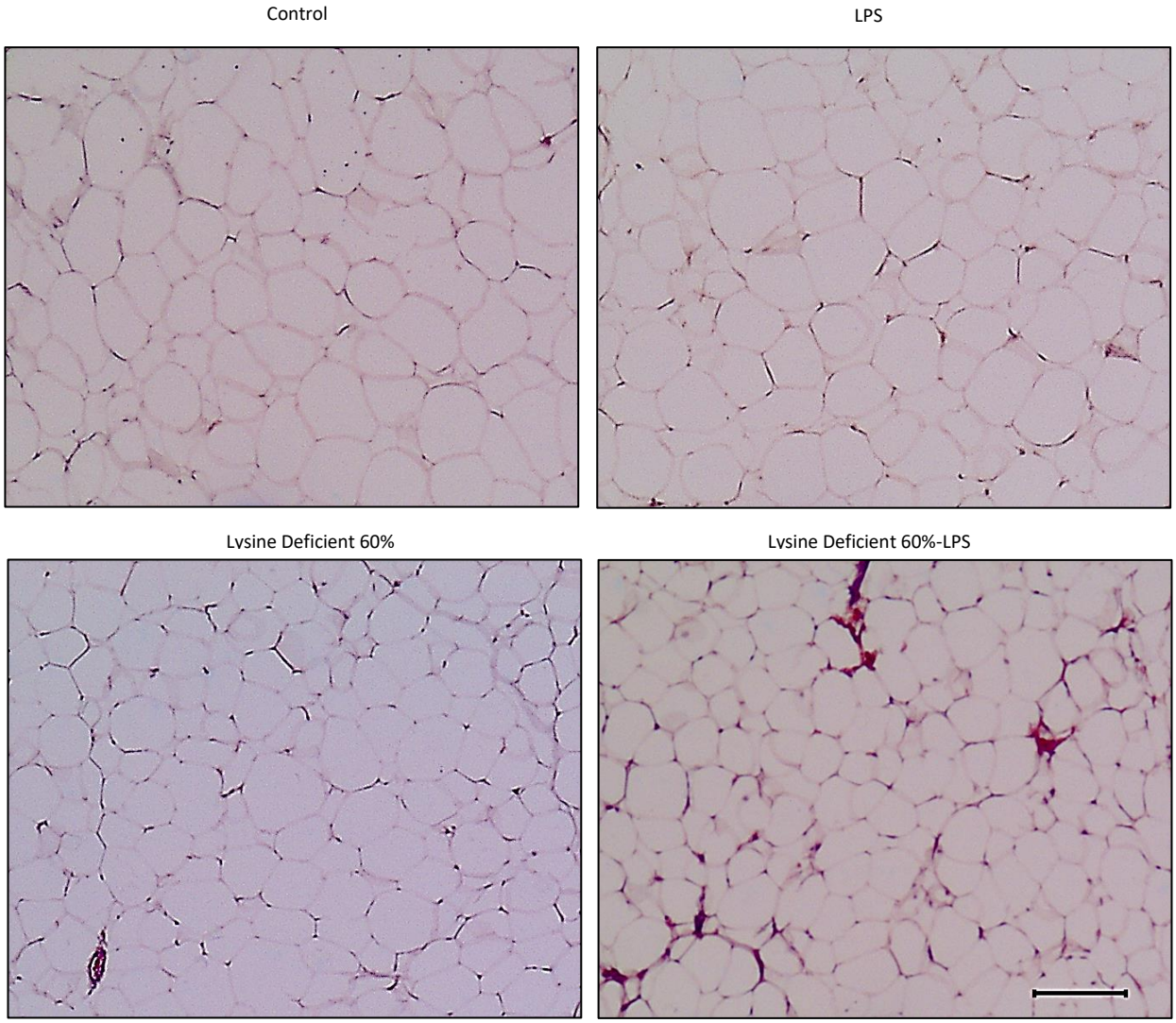

Figure 12. Representative H&E staining of epididymal fat pad from the different groups of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

Hematoxylin and Eosin (H&E) staining was done on sections of the epidydimal fat pad of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge. Images were taken at 4x magnification under a light microscope. Scale bar represents 100 µm.

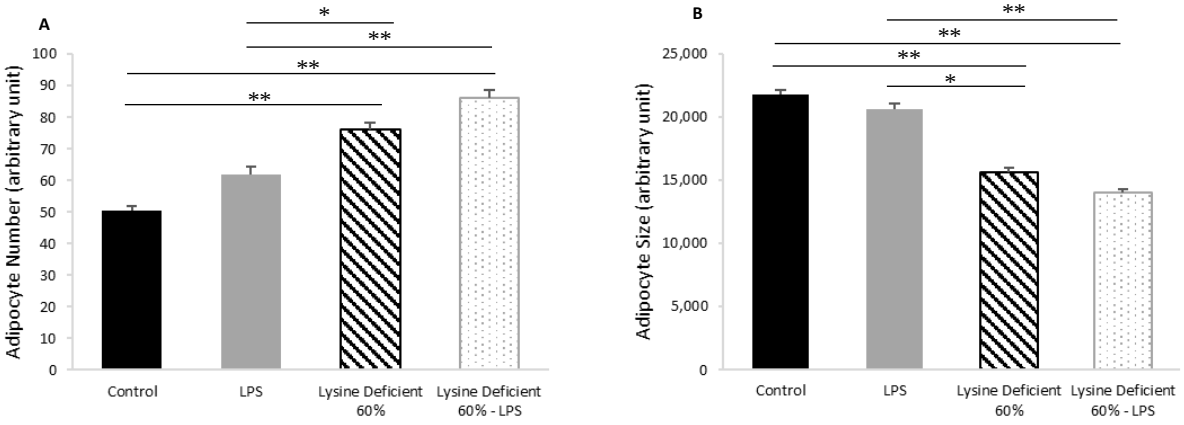

Figure 13. Epididymal adipocyte number and size in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

Hematoxylin and Eosin (H&E) staining was done on sections of the epidydimal fat pad of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge. Images were taken at 4x magnification under a light microscope (n=5 per group). The size of adipocytes was estimated using ImageJ software, and quantification was done manually for a set area in all slides.

Data are expressed as mean±SEM and were analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01).

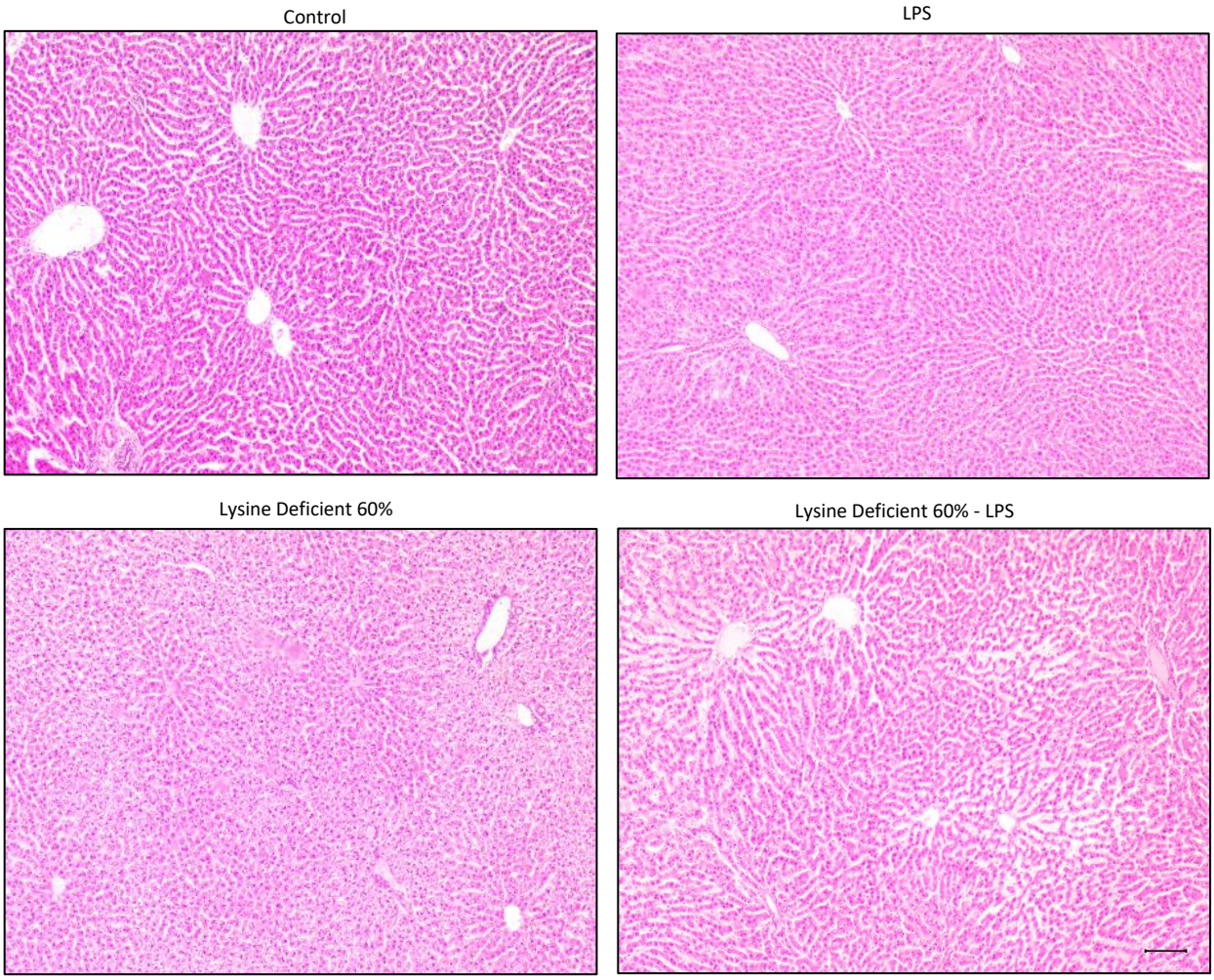

Figure 14. Representative H&E staining of liver sections from the different groups of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

Histological appearance of liver sections stained with Hematoxylin and Eosin at 4x magnification power in rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=5 per group). Scale bar represents 100 µm.

3. Blood

a. Glycemic profile

An IPGTT was performed on four rats per group. The glycemic response fluctuated between groups at all time points, as shown in Figure 15A. Despite the clear trend in the glycemic response, the AUC did not reach statistical significance, and values were comparable among groups (*p*-value=0.099) (Figure 15B). Nevertheless, the GLM analysis revealed that lysine deficiency dampened a normal glycemic response (*p*-value=0.024); however, the decrease due to the LPS challenge was not statistically significant (*p*-value=0.321) (Figure 15B).

In addition, no variations were identified between groups for fasting plasma glucose (*p*-value=0.389), insulin (*p*-value=0.595), GLP-1 (*p*-value=0.774), and HOMA-IR (*p*-value=0.645) (Figures 16).

b. Lipid profile and pH-mediated parameters

Lysine deficiency altered blood lipids significantly (Table 9). It drastically dropped plasma levels of triglyceride by half, total cholesterol by around 15%, and HDL cholesterol by 8%. LDL-cholesterol was shown to increase with lysine deficiency, yet the rise was not statistically significant. In contrast, LPS increased triglycerides by 20 mg/dl but did not affect any types of cholesterol.

Interestingly, lysine deficiency was associated with a remarkable upsurge in blood chloride but not sodium or lactate. pH-mediated parameters did not vary with LPS administration (Table 9).

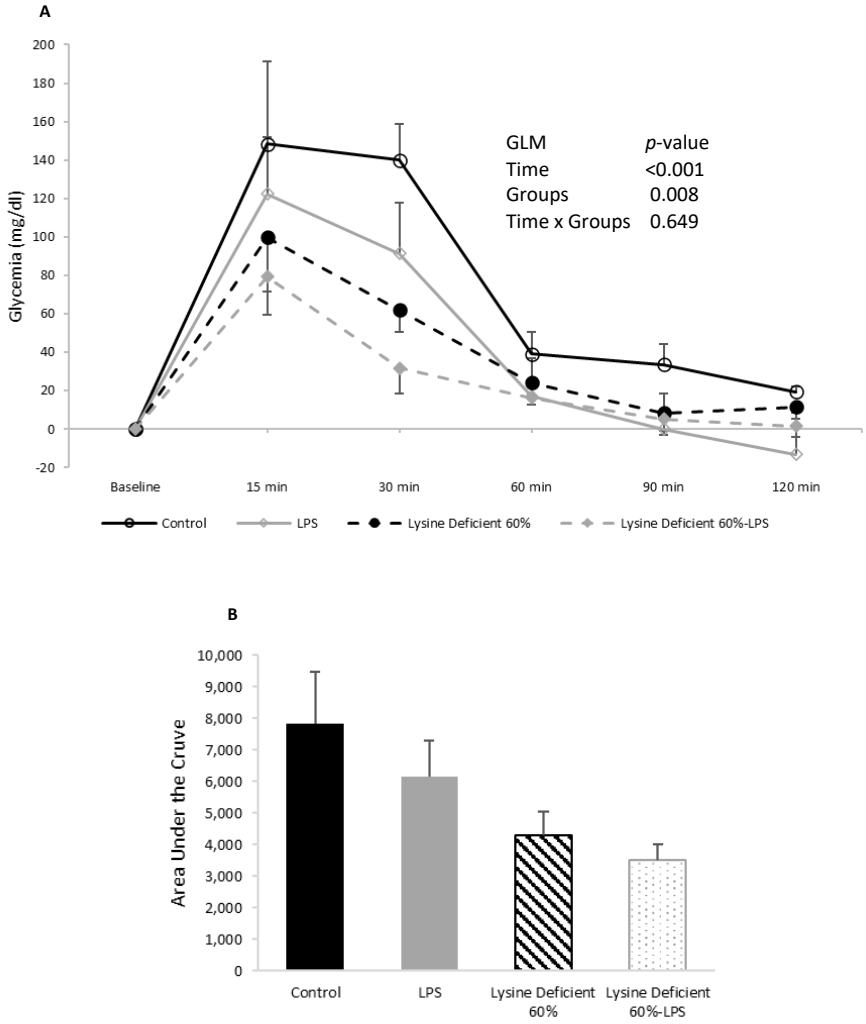

Figure 15. Intraperitoneal glucose tolerance test (IPGTT) of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Line chart plotting the glycemic values at each time point of the IPGTT, and (B) Area under the curve of the IPGTT in rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=4 per group).

Data are expressed as mean±SEM. General linear model (GLM) was used to analyze the differences of glycemic response in groups over 120 minutes, considering the two fixed factors (Time and Groups) and their interaction (Time x Groups). Also, one-way ANOVA was used to detect the difference between groups.

Statistical significance was set at p-value<0.05.

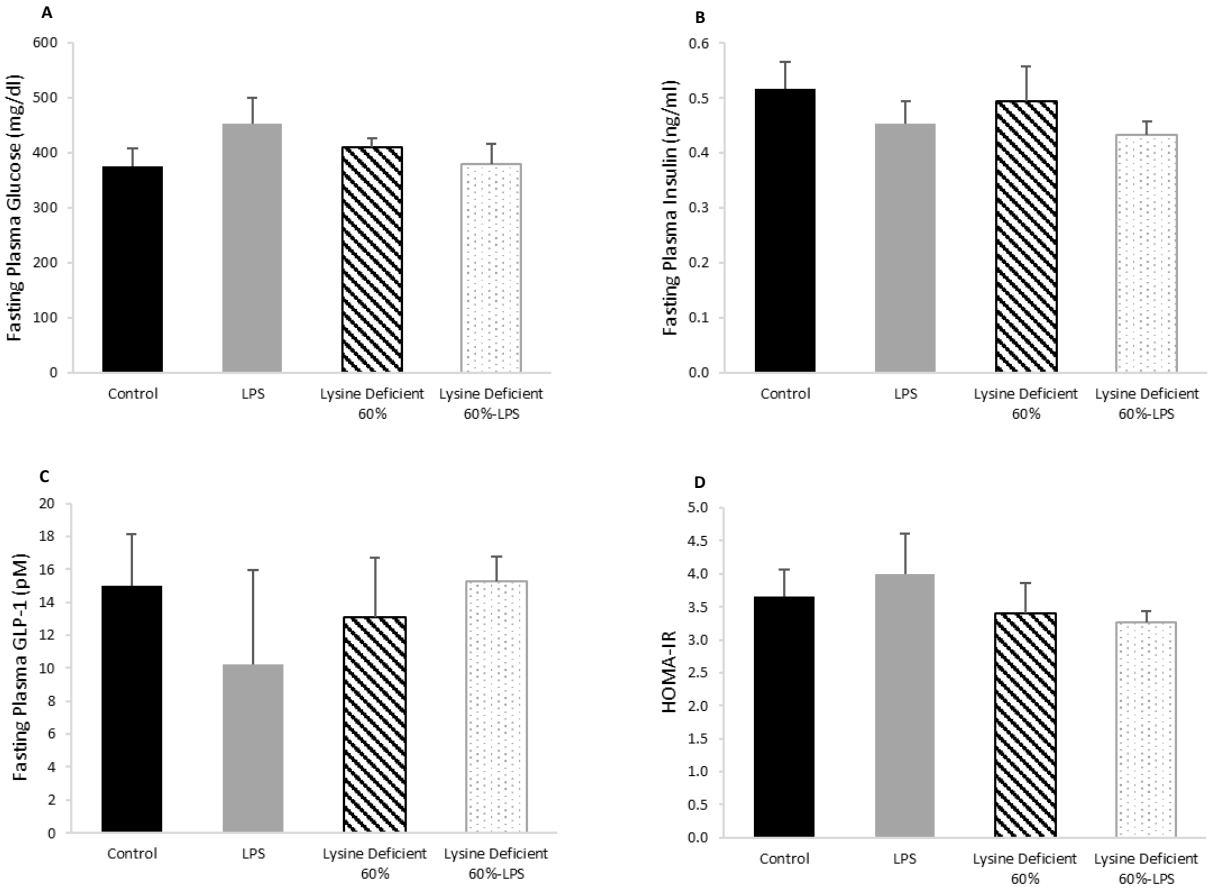

Figure 16. Glycemic profile of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Fasting plasma glucose, (B) Fasting plasma insulin, (C) Fasting plasma GLP-1, and (D) HOMA-IR index were measured at endpoint in rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n= 8 per group).

Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups.

Statistical significance was set at p-value<0.05.

Table 9. Fasting plasma lipid profile and pH-medicated parameters of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

	Control	LPS	Lysine Deficient 60%	Lysine Deficient 60%-LPS	*ANOVA* *p*-value
Lipid profile					
Triglyceride (mg/dl)	82.0±14.6[a]	112.0±44.5[b]	40.6±6.9[c]	38.7±6.2[c]	**<0.001**
Total Cholesterol (mg/dl)	91.3±6.6[ac]	96.8±16.6[a]	76.5±12.8[b]	82.0±6.1[bc]	**0.009**
HDL (mg/dl)	69.7±4.5[a]	71.5±11.3[a]	54.4±6.8[b]	65.0±5.2[a]	**<0.001**
LDL (mg/dl)	5.3±4.1	8.0±5.4	11.0±4.8	8.8±2.0	0.109
pH-mediated parameters					
Sodium (mmol/L)	136.6±1.2	136.4±0.8	135.6±1.3	135.7±1.6	0.302
Chloride (mmol/L)	92.3±1.3[a]	91.6±0.8[a]	97.8±2.4[b]	96.4±1.6[b]	**<0.001**
Lactate (mmol/L)	8.7±2.1	10.1±1.7	10.1±0.9	9.5±2.5	0.409

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons (n=8 per group). Means with the same letter are not significantly different (p-value>0.05).
Statistical significance was set at p-value<0.05.

c. Vasopressin

Plasma vasopressin was different among groups (*p*-value=0.001), with a distinctive increase in its level by around 27% in the LPS group compared to the control. Other groups presented a similar vasopressin profile (Figure 17).

d. Amino acid

Plasma free amino acid concentrations were determined by HPLC. As shown in Table 10, all essential amino acids, along with glycine, serine, and tyrosine were different with lysine deficiency. Plasma lysine concentration of the lysine deficient groups decreased by 50% to 60%, confirming a lower intake. The GLM analysis affirmed that the existent variation in amino acid concentration was exclusively related to the content of lysine in the diet, and no direct effect of LPS (or interaction) was detected. It is worth noting that in the lysine-deficient groups, almost all amino acids decreased while histidine and serine increased by 50% and threonine by 2.5 folds.

Despite the changes in single amino acids among groups, the total concentration of essential amino acids was maintained. Similarly, the total concentration of non-essential amino acids was comparable between the groups. Representative chromatograms are displayed in Figure 18.

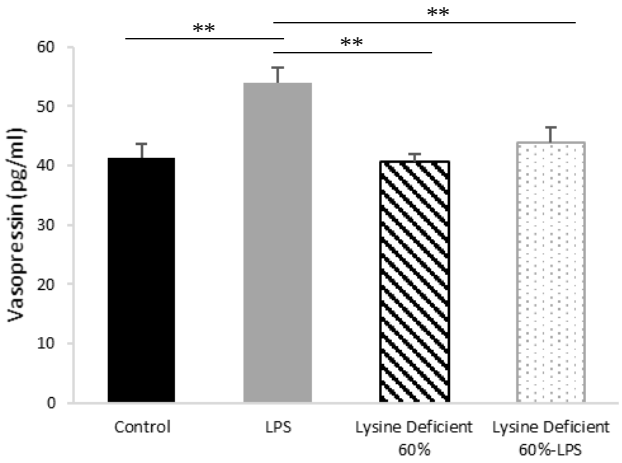

Figure 17. Plasma vasopressin levels in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

Plasma vasopressin levels of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=6 per group).

Data are expressed as the mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (** p-value<0.01).

Table 10. Plasma amino acid levels in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

Amino Acids (µmoles/L)	Control	LPS	Lysine Deficient 60%	Lysine Deficient 60%-LPS	*ANOVA* *p*-value
Essential amino acids					
Histidine	86.9±14.0[a]	88.4±20.8[a]	127.1±30.6[b]	129.3±21.9[b]	**0.001**
Isoleucine	109.6±20.1[a]	116.6±22.9[a]	85.5±19.1[b]	79.2±16.6[b]	**0.003**
Leucine	197.1±33.1[ab]	207.5±29.9[a]	168.0±29.9[bc]	152.8±21.4[c]	**0.006**
Lysine	452.0±131.7[a]	472.4±47.1[a]	220.6±56.8[b]	190.5±27.8[b]	**<0.001**
Methionine	55.5±15.8[a]	56.8±7.9[a]	34.8±13.6[b]	31.0±4.2[b]	**<0.001**
Phenylalanine	88.4±12.2[a]	90.5±10.5[a]	60.4±10.1[b]	51.8±7.1[b]	**<0.001**
Threonine	243.6±42.7[a]	241.5±48.3[a]	653.8±314.8[b]	587.2±171.4[b]	**<0.001**
Tryptophan	78.9±21.6[a]	77.5±14.0[a]	40.6±11.6[b]	46.8±12.4[b]	**<0.001**
Valine	211.8±29.1[a]	209.9±26.4[a]	161.6±29.6[b]	146.7±17.9[b]	**<0.001**
Total	1523.8±258.1	1561.0±163.8	1552.4±394.0	1415.3±195.1	0.759
Non-essential amino acids					
Alanine	748.1±115.2	784.6±108.8	833.6±169.6	781.3±175.9	0.696
Arginine	88.4± 55.5	98.1± 67.0	88.9± 36.9	85.5± 29.8	0.967
Asparagine	59.3±18.5	59.9±15.7	49.9±11.7	46.4±8.0	0.277
Citrulline	103.8±14.1	102.0±9.1	101.9±13.1	90.2±13.7	0.217
Glutamic acid	346.4±109.6	403.4±95.2	366.8±114.2	292.0±80.0	0.261
Glutamine	766.4±147.0	766.4±92.9	917.4±176.2	885.5±134.5	0.090
Glycine	362.9±67.7[ab]	435.8±91.4[b]	318.0±61.1[a]	328.0±12.9[ab]	**0.011**
Ornithine	144.9±83.0	137.6±82.0	104.9±60.9	82.0±38.6	0.326
Serine	405.6 ±40.4[a]	399.5±64.3[a]	586.5±128.9[b]	563.7±82.0[b]	**<0.001**
Taurine	260.0±76.8	300.4±59.1	292.8±82.8	276.7±29.6	0.646
Tyrosine	85.5±36.8[a]	87.4±12.1[a]	65.5±15.7[ab]	40.5±15.1[b]	**0.002**
Total	3371.1±362.7	3575.0±351.1	3719.8±512.0	3409.3±401.9	0.343
Overall total	4895.9±579.4	5137.0±457.9	5273.1±857.8	4825.7±507.9	0.504

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons (n=8 per group). Means with the same letter are not significantly different (p-value>0.05).
Statistical significance was set at p-value<0.05.

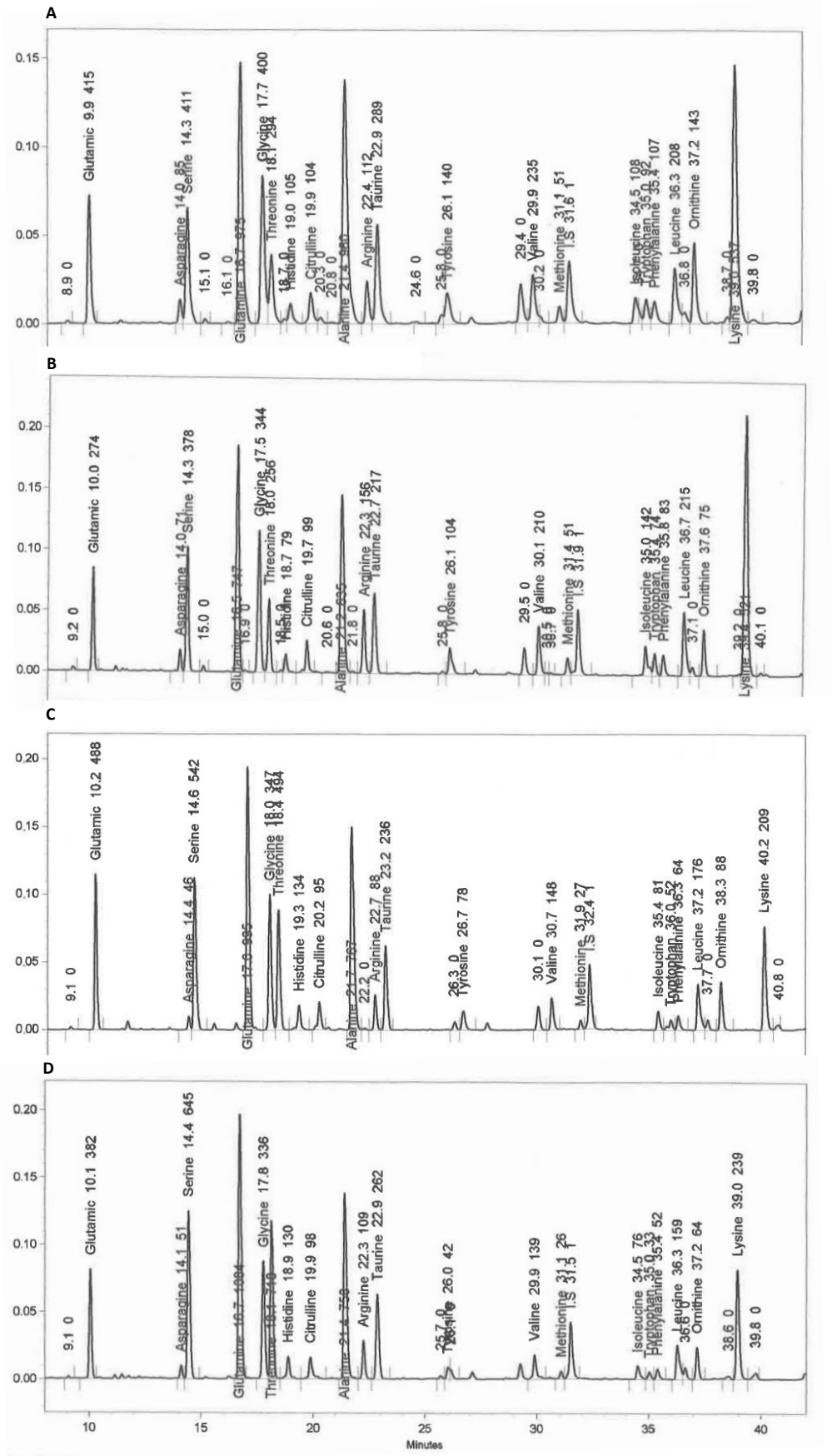

Figure 18. HPLC chromatograms of plasma amino acids in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

A sample amino acid chromatography in plasma of the (A) Control, (B) LPS, (C) Lysine Deficient 60%, and (D) Lysine Deficient 60%-LPS group.

4. Albumin

The hepatic mRNA expression of albumin was different in groups (*p*-value=0.005). A decrease was detected in the liver of lysine-deficient rats, and no influence of LPS on this result was found.

This was confirmed by the drop in plasma albumin levels among the same groups (*p*-value <0.001). Plasma albumin concentration dropped by around 20% with lysine deficiency. Interestingly, a slight increase of around 10% in circulating albumin was observed among rats of the Lysine Deficient-LPS compared to the Lysine Deficient group. This increment associated with LPS was not significant among rats fed a control diet (Figure 19).

5. CRP

The relative mRNA expression of CRP in the liver was similar among groups (*p*-value=0.062). Nevertheless, its hepatic protein expression was different (*p*-value=0.006). CRP expression increased by around 2.5 folds in the Lysine Deficient compared to the Control group. Surprisingly, our data indicated a decreasing effect associated with LPS administration, though the decrease was not significant when rats were fed a control diet (Figure 20).

On the other hand, the differences in the epididymal fat pad mRNA and protein expression of CRP were insignificant (*p*-values=0.555 for the mRNA and 0.436 for the protein) (Figure 21).

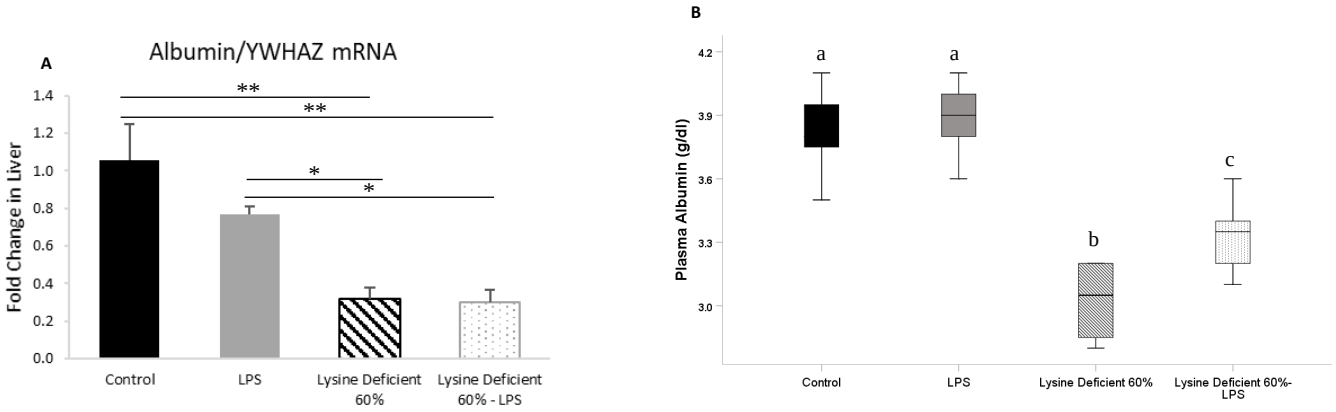

Figure 19. mRNA expression and plasma levels of albumin in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) qRTPCR result showing the relative mRNA expression of albumin in the liver (n=5 per group) and (B) Fasting plasma albumin levels (n=8 per group) of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge.

mRNA data are expressed as mean±SEM and plasma levels are expressed as median (the bar inside the box), the interquartile range (the box length), the minimum value (the lower bar cap) and the maximum value (the upper bar cap). Results were analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Boxes of groups sharing the same letter have statistically similar results.

Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01).

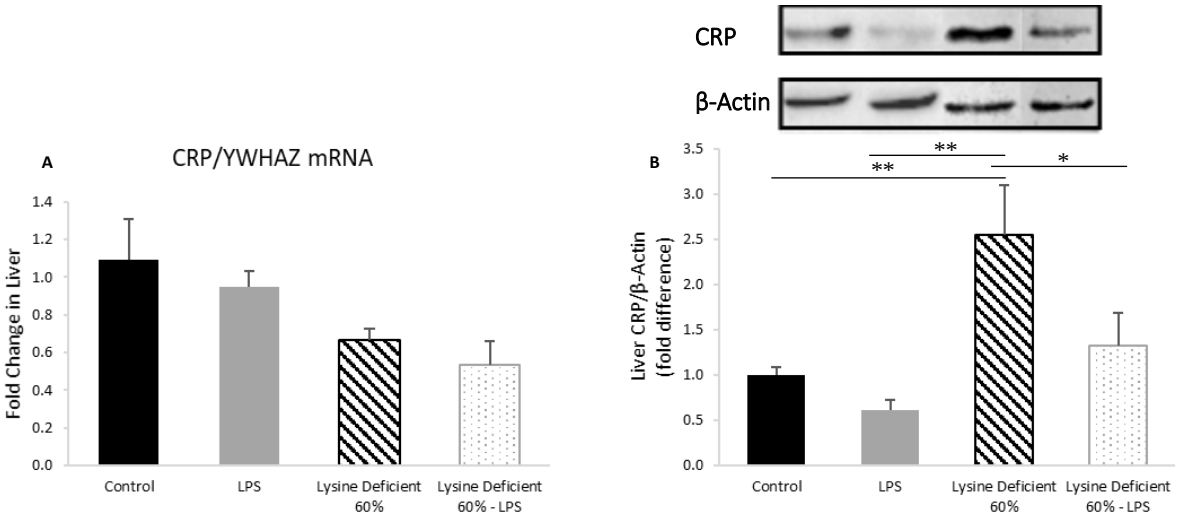

Figure 20. Hepatic mRNA and protein expressions of CRP in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) qRTPCR result showing the relative mRNA expression of CRP (n=5 per group) and (B) Western Blot result presenting protein CRP expression (n=8 per group) in the liver of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge.

Protein bands detected by Western blots were quantified with Image J software and normalized against β-Actin. Data are expressed as mean±SEM, and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01).

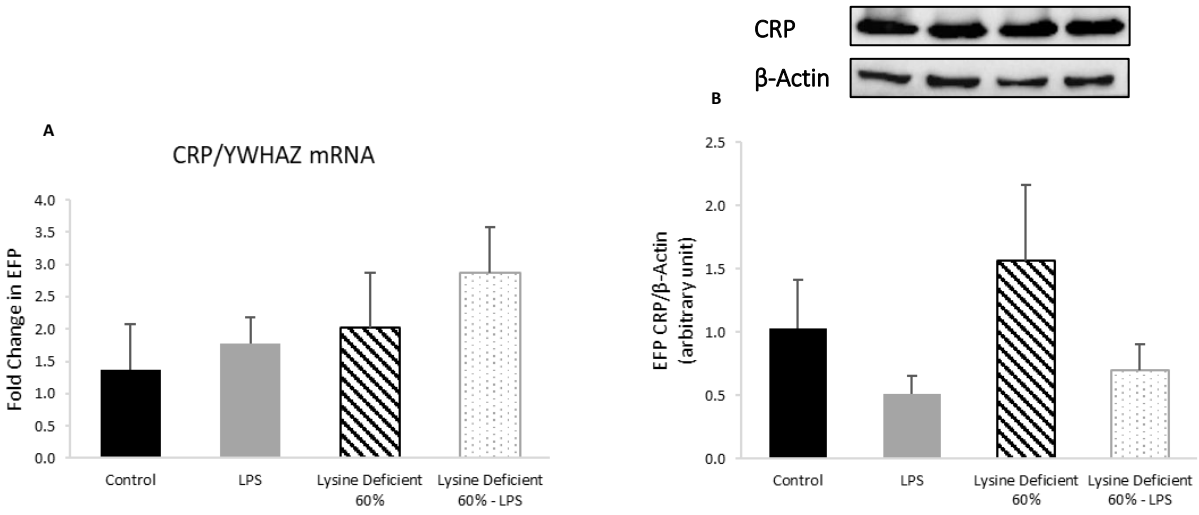

Figure 21. mRNA and protein expressions of CRP in the epididymal fat pad of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

EFP: Epididymal fat pad.

(A) qRTPCR result showing the relative mRNA expression of CRP (n=5 per group) and (B) Western Blot result presenting protein CRP expression (n=8 per group) in the epididymal fat pad of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge.

Protein bands detected by western blots were quantified with Image J software and normalized against β-Actin. Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05.

6. Other inflammatory markers

Gene expression to the housekeeping gene YWHAZ was assessed via qRT-PCR and different organs displayed varied profiles. In the liver, TLR4 (*p*-value=0.632), MyD88 (*p*-value=0.897), and IL-6 receptor (IL-6R) (*p*-value=0.624) mRNA expressions were not different among groups. Nonetheless, IL-1β had a higher expression in the LPS group than in all other groups (Figure 22). IL-6, TNF-α, and interferon γ (INF-γ) were not expressed in the liver.

In the epididymal fat pad, TLR4 (*p*-value=0.572), MyD88 (*p*-value=0.420), and IL-1β (*p*-value=0.002) had a similar profile to the one in the liver (Figure 23). In addition, IL-6, IL-6R, TNF-α, and INF-γ were not induced by the experimental groups at endpoint.

Regarding the spleen, TLR4 (*p*-value=0.050) and IL-6R (*p*-value=0.250) were similar among groups with a tendency of increment with LPS administration. This pattern was significant for MyD88 (*p*-value=0.015), IL-6 (*p*-value=0.001), IL-1β (*p*-value=0.011), and TNF-α (*p*-value=0.017) that were induced by LPS administration. Also, mRNA expression of INF-γ, showed no specific trend (*p*-value=0.074) (Figure 24).

Interestingly, lysine deficiency did not contribute to any of these results.

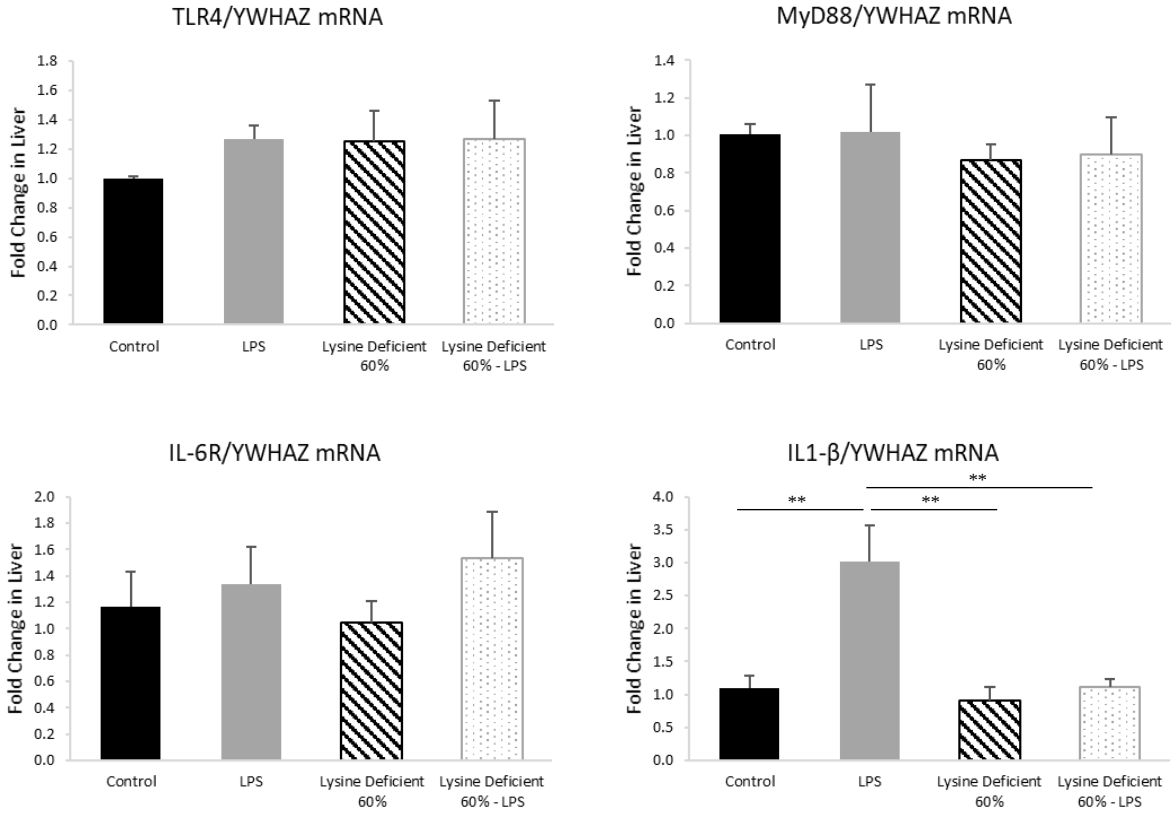

Figure 22. mRNA expression of TLR4, MyD88, IL-6R, and IL-1β in the liver of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

qRTPCR results showing the relative mRNA expression of TLR4, MyD88, IL-6R, and IL-1β in the liver of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=5 per group).

Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (** p-value<0.01).

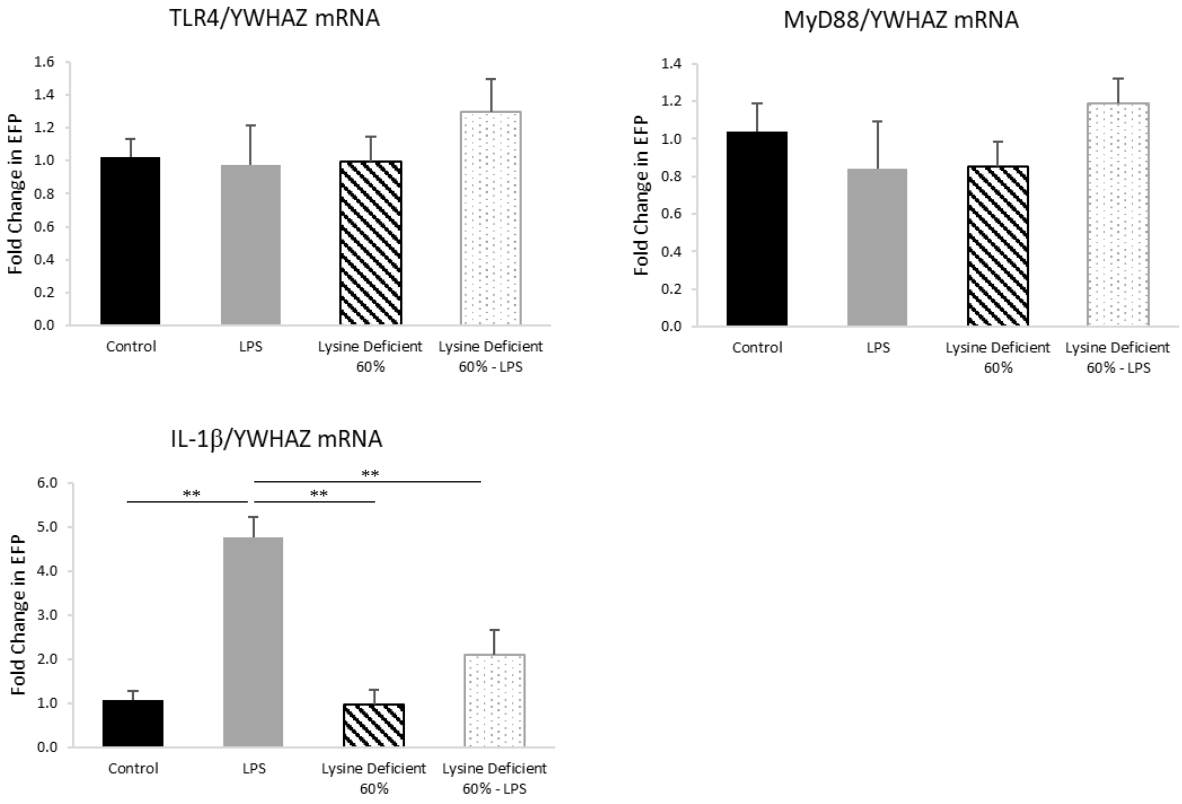

Figure 23. mRNA expression of TLR4, MyD88, and IL-1β in the epididymal fat pad of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

EFP: Epididymal fat pad.

qRTPCR results showing the relative mRNA expression of TLR4, MyD88, and IL-1β in the epididymal fat pad of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=5 per group).

Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (** p-value<0.01).

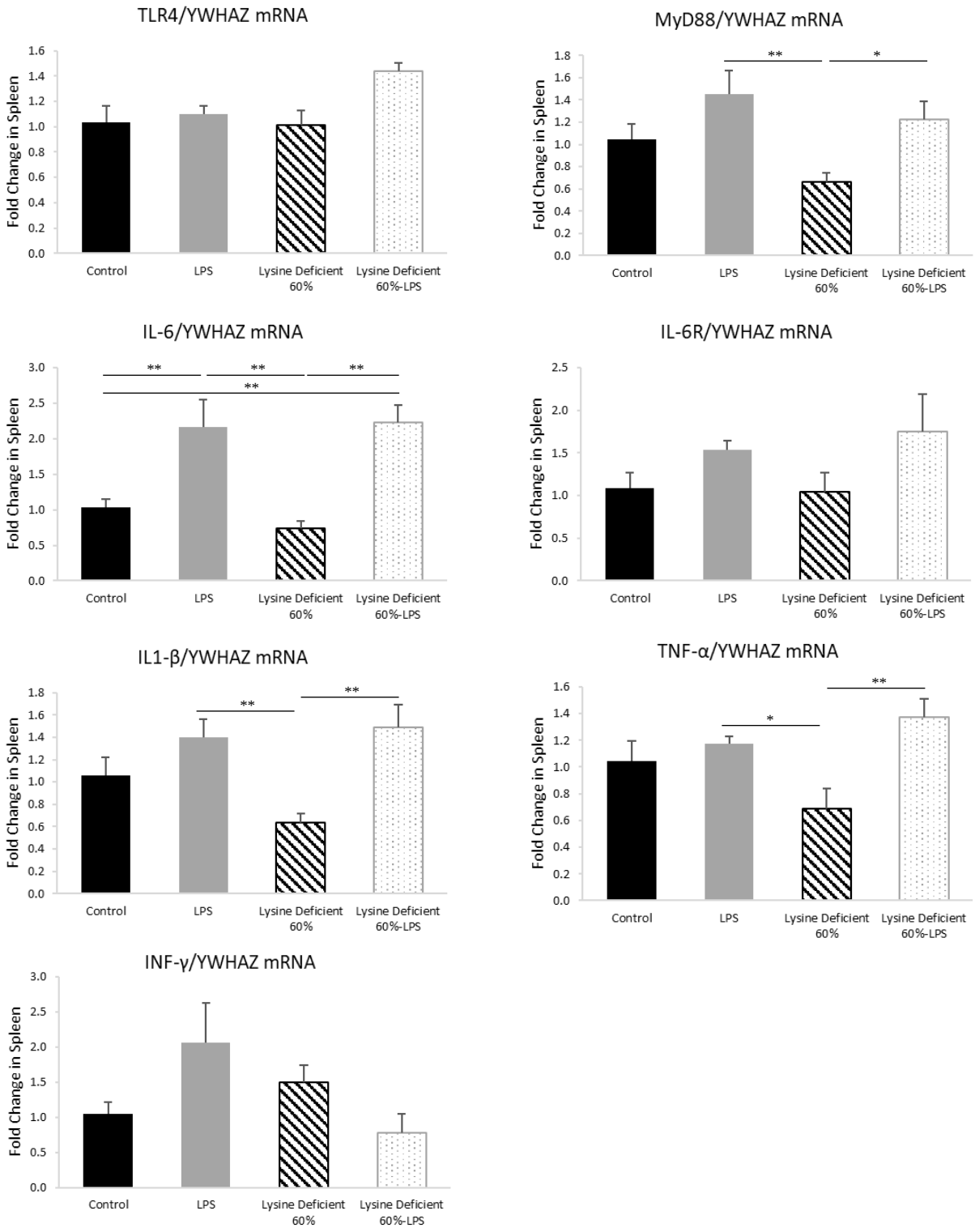

Figure 24. mRNA expression of TLR4, MyD88, IL-6, IL-6R, IL-1β, TNF-α, and INF-γ in the spleen of rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

qRTPCR results showing the relative mRNA expression of TLR4, MyD88, IL-6, IL-6R, IL-1β, TNF-α, and INF-γ in the spleen of rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=5 per group).

Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01).

7. Behavioral tests

The Hargreaves test was performed for pain assessment a week before euthanasia and showed no difference among the groups (*p*-value=0.501) (Figure 25).

Over three consecutive days, the OFT was completed, and results are displayed in Figures 26 to 29. The two-way repeated measures ANOVA with post hoc Fisher LSD revealed a difference related to the repetitive sessions for the total distance traveled that increased in session 3 (*p*-value=0.041), the distance traveled in the peripheral areas that decreased in sessions 2 and 3 (*p*-value=0.002), and the percent time spent in corners (*p*-value=0.011). However, the statistical test failed to disclose differences attributed to groups or session x group interaction (Figure 26). Nonetheless, when sessions were analyzed separately, session 1 affirmed the evidence of an increase in the total distance traveled (*p*-value=0.010) and distance traveled in the periphery (*p*-value=0.008) among the Lysine Deficient 60%-LPS group (Figure 27). No effect of LPS administration was observed when the diet was complete (control diet). The increase in the distance was not noticed in the internal (*p*-value=0.804) or central (*p*-value=0.402) areas. Also, rats who ate a lysine-deficient diet spent more time in the corner, irrespective of the presence of LPS (Figure 27). Interestingly, the GLM analysis attributed the effect on distance and time to lysine deficiency (*p*-value<0.01 for all) but not LPS. While the percentage of time spent in the corner was increased with lysine deficiency, no effect between the groups was detected in session 1 for the fraction of time spent in the peripheral (*p*-value=0.954), internal (*p*-value=0.955) or central areas (*p*-value=0.510). Representative trajectories taken during the first session of the OFT are displayed in Figure 30.

All parameters were similar among groups in session 2 (Figure 28) and session 3 (Figure 29).

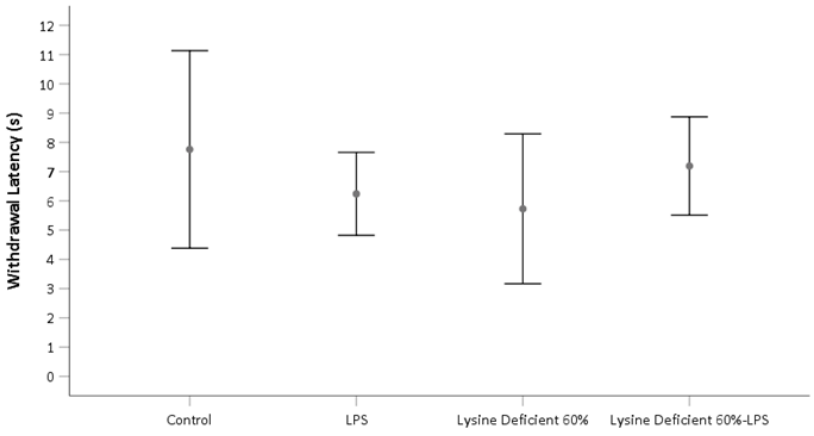

Figure 25. Thermal pain sensation in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

This heat stimulus was applied thrice per rat, separated by an interval of 5 minutes. The average of the two closest values was reported. Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05.

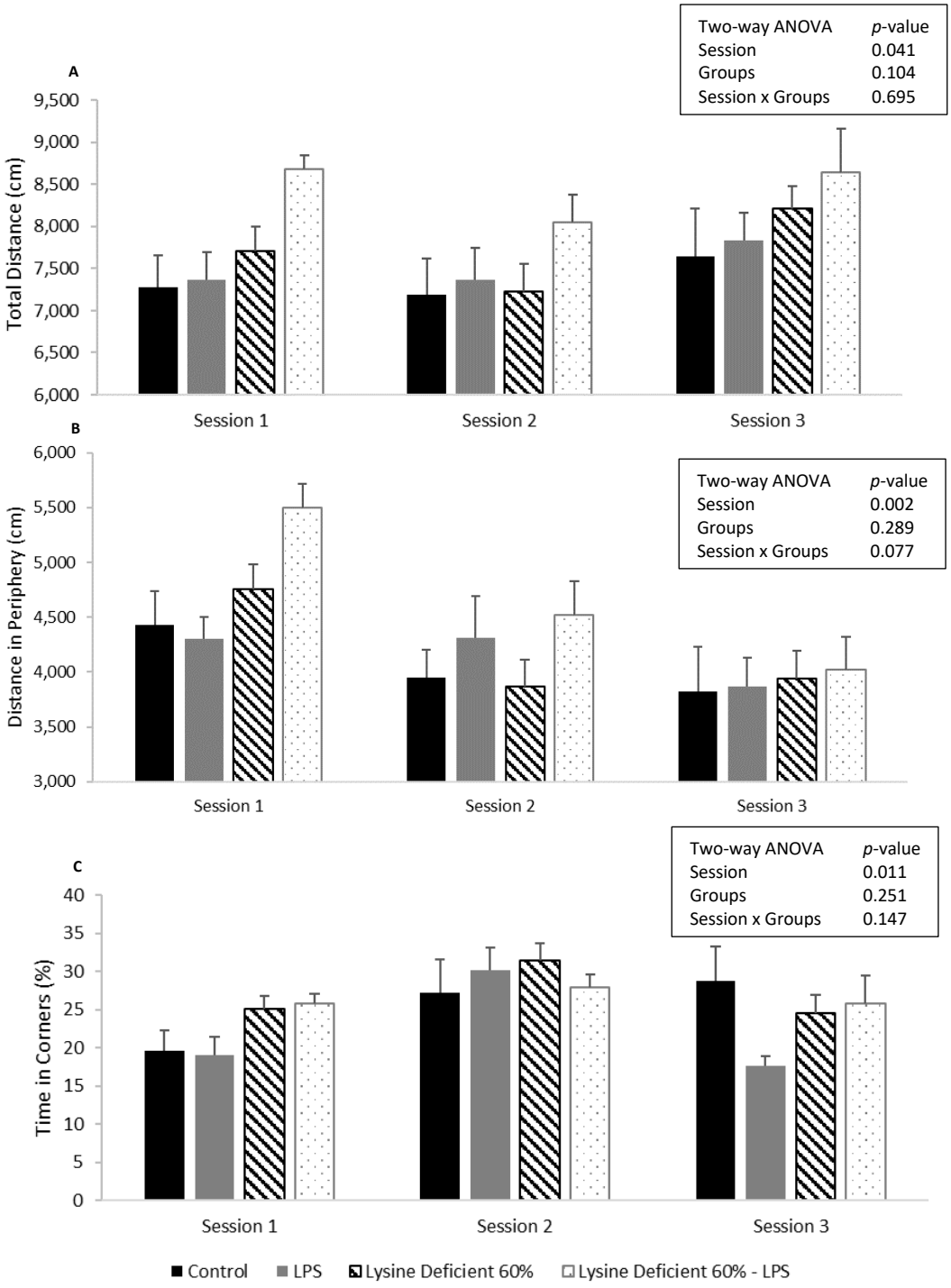

Figure 26. Total distance, distance in the periphery, and time spent in corners over the three sessions of the Open Field Test in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Total distance traveled over the three consecutive sessions, (B) Distance traveled in the peripheral area over the three consecutive sessions, and (C) The percent time spent in corners over the three consecutive sessions by rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group).

Data are expressed as mean±SEM and described using SMART software. Analysis was done by a two-way repeated measures ANOVA to detect variation of means in groups over repeated sessions among groups followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05.

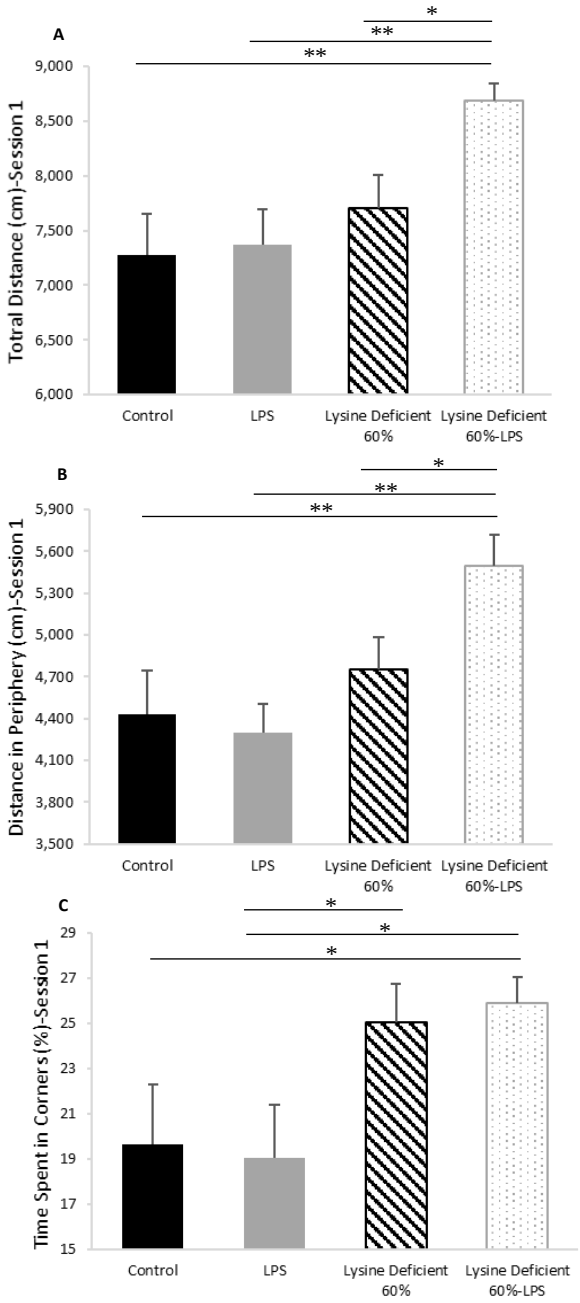

Figure 27. Total distance, distance in the periphery, and time spent in corners in session 1 of the Open Field Test in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Total distance traveled, (B) Distance traveled in the peripheral area, and (C) The percent time spent in corners, during session 1, by rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group).

Data are expressed as mean±SEM and described using SMART software. Analysis was done by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01).

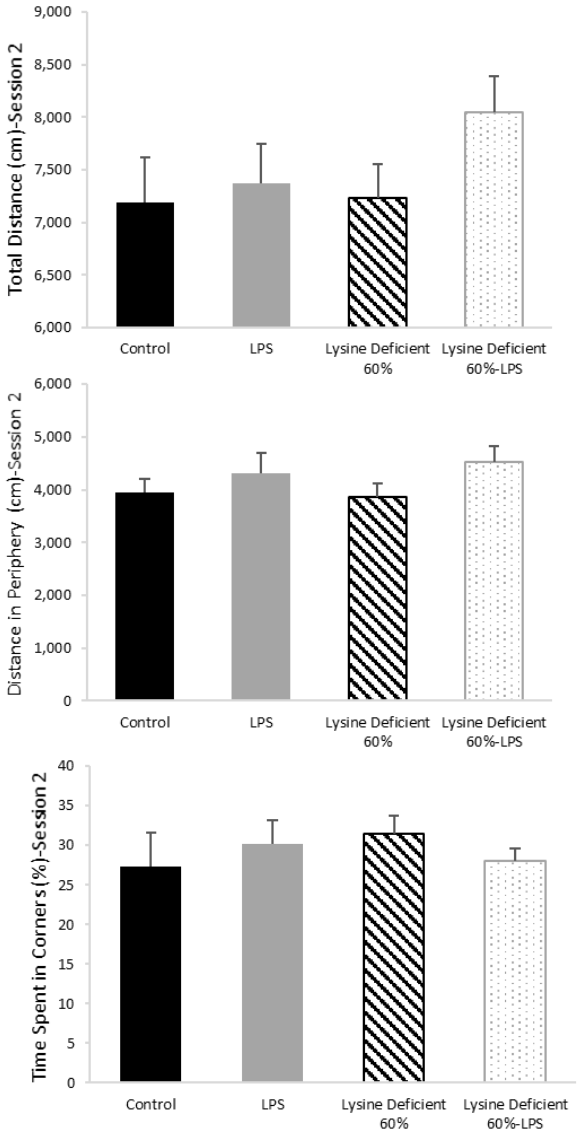

Figure 28. Total distance, distance in the periphery, and time spent in corners in session 2 of the Open Field Test in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Total distance traveled, (B) Distance traveled in the peripheral area, and (C) The percent time spent in corners, during session 1, by rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group).

Data are expressed as mean±SEM and described using SMART software. Analysis was done by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05.

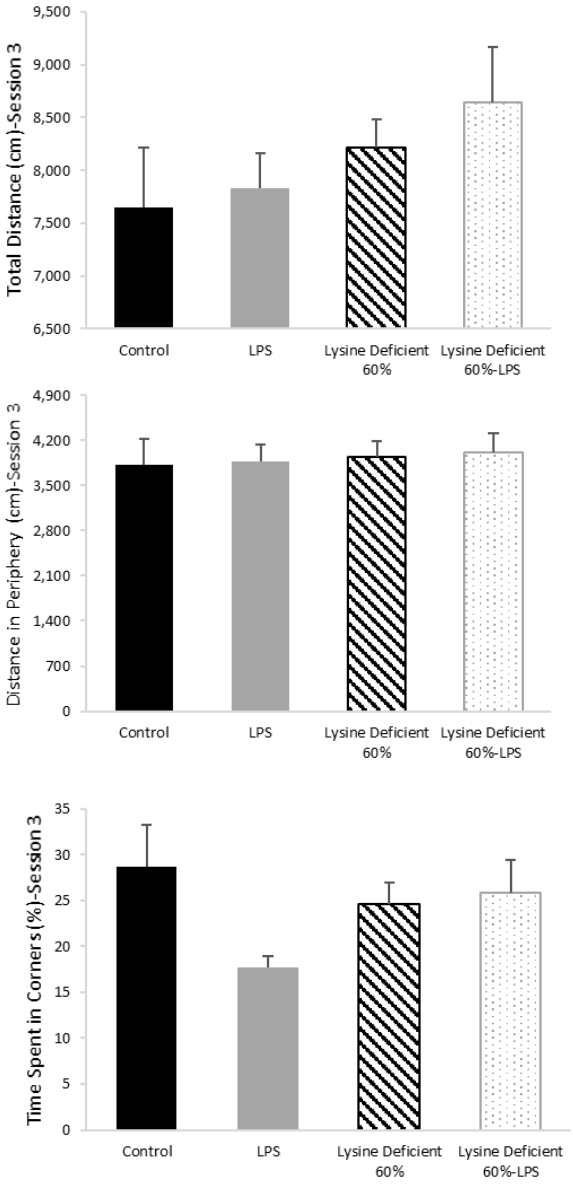

Figure 29. Total distance, distance in the periphery, and time spent in corners in session 3 of the Open Field Test in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

(A) Total distance traveled, (B) Distance traveled in the peripheral area, and (C) The percent time spent in corners, during session 1, by rats fed a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge (n=8 per group).

Data are expressed as mean±SEM and described using SMART software. Analysis was done by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05

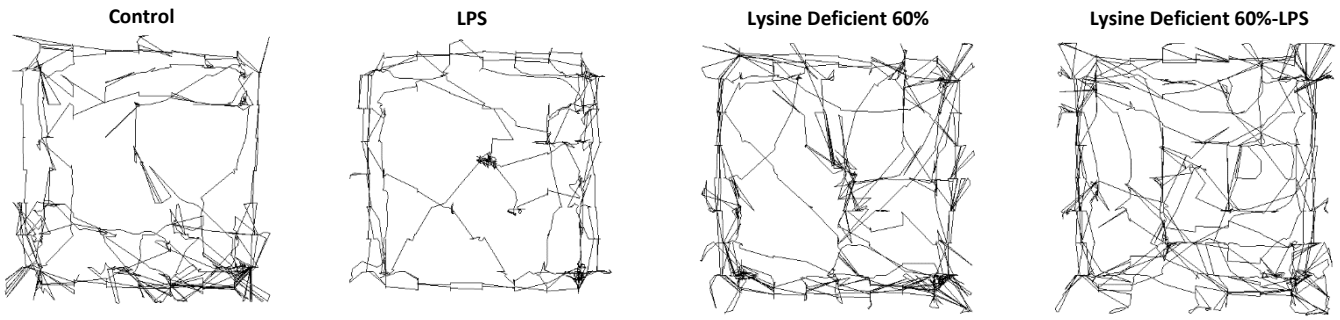

Figure 30. Sample trajectory of the group during session 1 of the Open Field Test in rats maintained on a control diet or a 60% lysine-deficient diet in the presence or absence of LPS challenge

The trajectories were recorded for 5 minutes via a video camera connected to the tracking software Smart.

B. Experiment 2

Investigating the effect of 30% lysine deficiency in diet on the body compartment, inflammatory profile, and behavior in young male Sprague Dawley rats.

1. Body composition, food intake, and energy parameters

Differences in body composition and compartments were less evident, with 30% lysine deficiency. Weight changes were similar between the control and unchallenged deficient group, with a final gain of around 150 g. In contrast, weight gain was 20 g lower at endpoint in the LPS group (Figure 31A).

Three weeks after the startup of the experiment, fat accretion (p-value=0.638) (Figure 31B) and extracellular water (p-value=0.273) (Figure 31D) were similar among groups. The only difference resides in the lean stores (p-value=0.004), in which lysine-deficient groups (with or without LPS) had 20% less lean mass gain than the control group. No effect was attributed to LPS administration.

Differences were not detected for food (p-value=0.610) and water (p-value=0.118) intakes (Figure 32), as well as energy efficiency (p-value=0.448) and expenditure (p-value=0.949) (Figure 33).

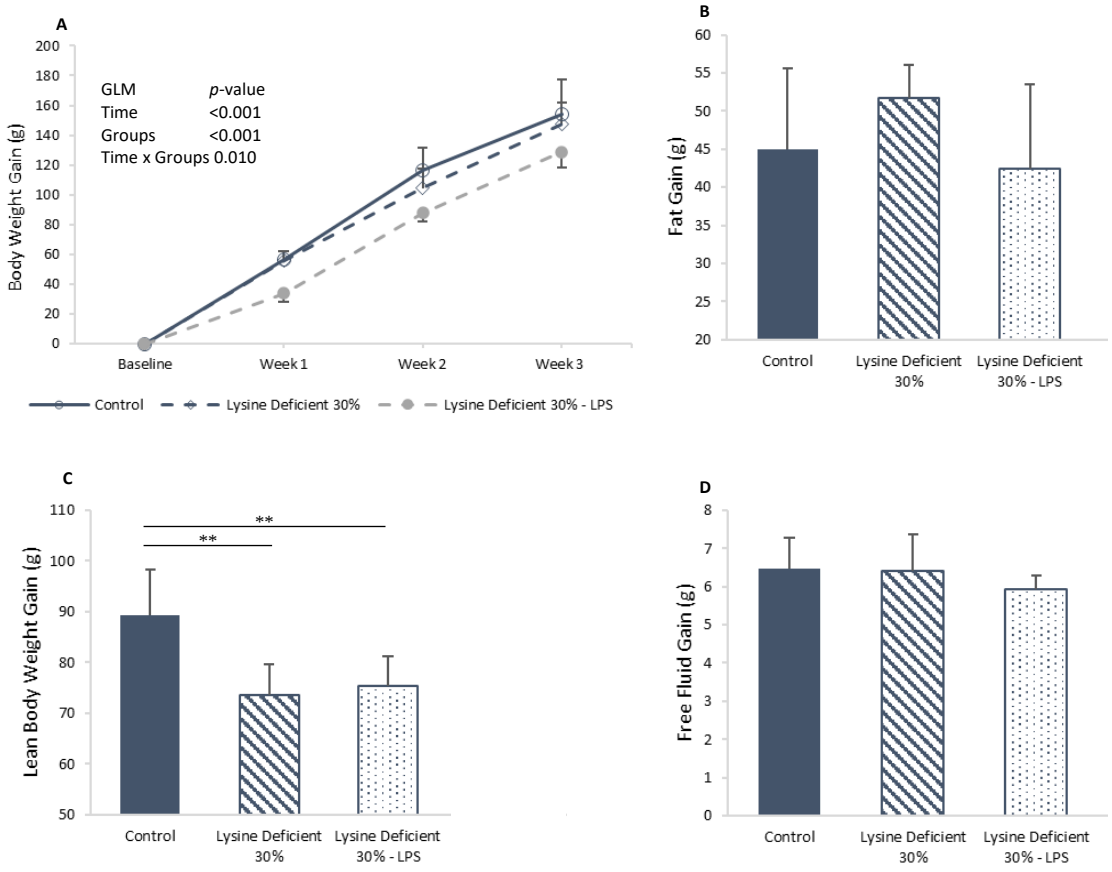

Figure 31. Body weight and composition of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

A technical problem occurred in week 4 of the experiment putting the TD-NMR Minispec LF110 down. Hence, the body composition analysis of Experiment 2 was evaluated for three weeks.

(A) Body weight gain from baseline for 3 weeks, (B) Fat gain from baseline at endpoint, (C) Lean mass gain from baseline at endpoint, and (D) Body free fluid gain from baseline at endpoint of rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as the mean±SD. General linear model (GLM) was used to analyze the effect of different parameters across the experimental period, considering the two fixed factors (Time and Groups) and their interaction (Time x Groups).

Statistical significance was set at p-value<0.05 (** p-value<0.01).

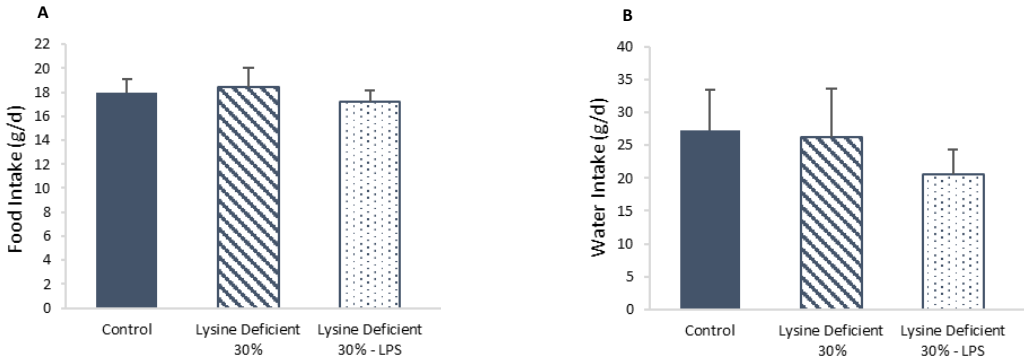

Figure 32. Food and water intake of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) Food intake and (B) Water intake per day of rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at *p*-value<0.05.

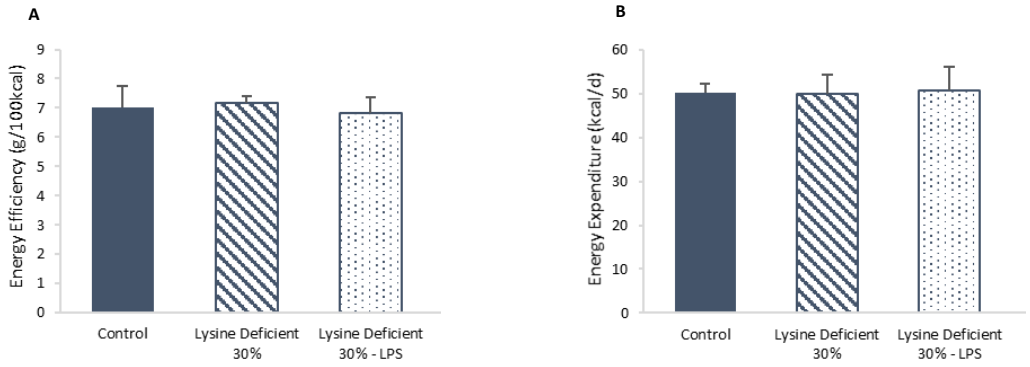

Figure 33. Energy balance of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) Energy efficiency and (B) Energy expenditure per day of rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at *p*-value<0.05.

2. Organs

Absolute weights of the organs are written in Table 11, and adjusted weights to 100 g body weight are displayed in Figure 33. The spleen weight was reduced with lysine deficiency and increased with LPS. Yet, no difference was attributed to lysine deficiency when adjustment was made. Spleens of LPS-challenged rats were significantly enlarged by around 50%.

In contrast, LPS administration produced a 25% decrease in the epididymal fat pad, irrespective of the weight adjustment. The absolute liver weight was reduced with lysine deficiency. However, liver weight/100 g body weight was shown to slightly increase in the challenged group compared to the unchallenged deficient group. Variations in liver weight were not related to triglyceride accumulation which was similar between groups (*p*-value=0.294) (Figure 34).

Microscopic images of H&E-stained epididymal fat sections are presented in Figure 35. Both adipocyte size and number differed between groups (*p*-value=0.010 and *p*-value=0.001). Lysine deficiency decreased the size of the adipocytes (*p*-value=0.018), while its effect on the number was insignificant. On the other hand, LPS injections increased the count but did not impact the area significantly (Figure 36).

Liver sections were also stained with H&E (Figure 37). No significant morphological changes or prominent immune cell infiltration were described.

Table 11. Weight of the organs of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

Organs (g)	Control	Lysine Deficient 30%	Lysine Deficient 30%-LPS	*ANOVA* *p*-value
Spleen	0.97±0.12[a]	0.81±0.09[b]	1.14±0.09[c]	**<0.001**
Epididymal Fat Pad	7.08±1.69[a]	5.45±1.48[ab]	4.19±0.76[b]	**0.006**
Liver	18.03±1.68[a]	14.77±1.74[b]	15.72±1.09[b]	**0.012**
Final Body Weight*	425.9±34.6[a]	378.0±24.8[b]	367.5±22.6[b]	**0.006**

* Final body weight was measured at week 4 before euthanasia.

Weights of the organs were assessed in rats fed a control diet (n=4) or a 30% lysine deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Means with the same letter are not significantly different (p-value>0.05).

Statistical significance was set at p-value<0.05.

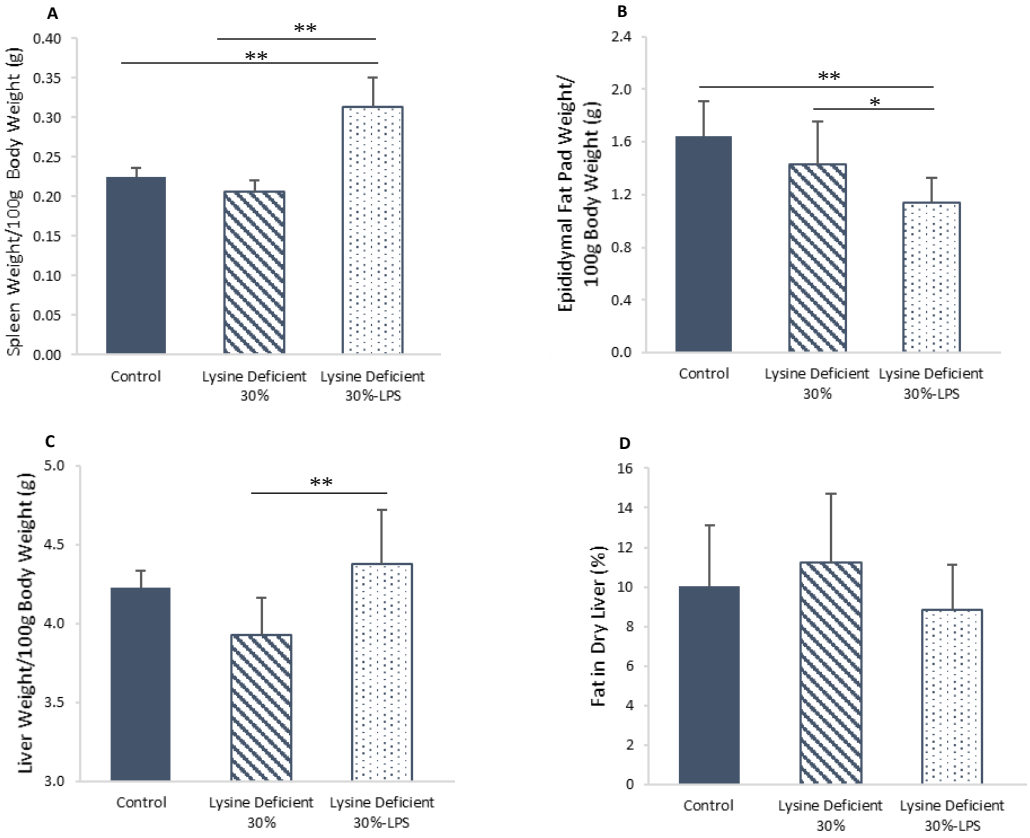

Figure 34. Adjusted organs weights and liver fat content of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) Spleen, (B) Epididymal fat pad, and (C) Liver weights per 100 g of body weight, and (D) Percentage of fat content in dry liver of rats fed a control diet (n=4) or a 30% lysine deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data were presented as mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05. (* p-value<0.05, ** p-value<0.01).

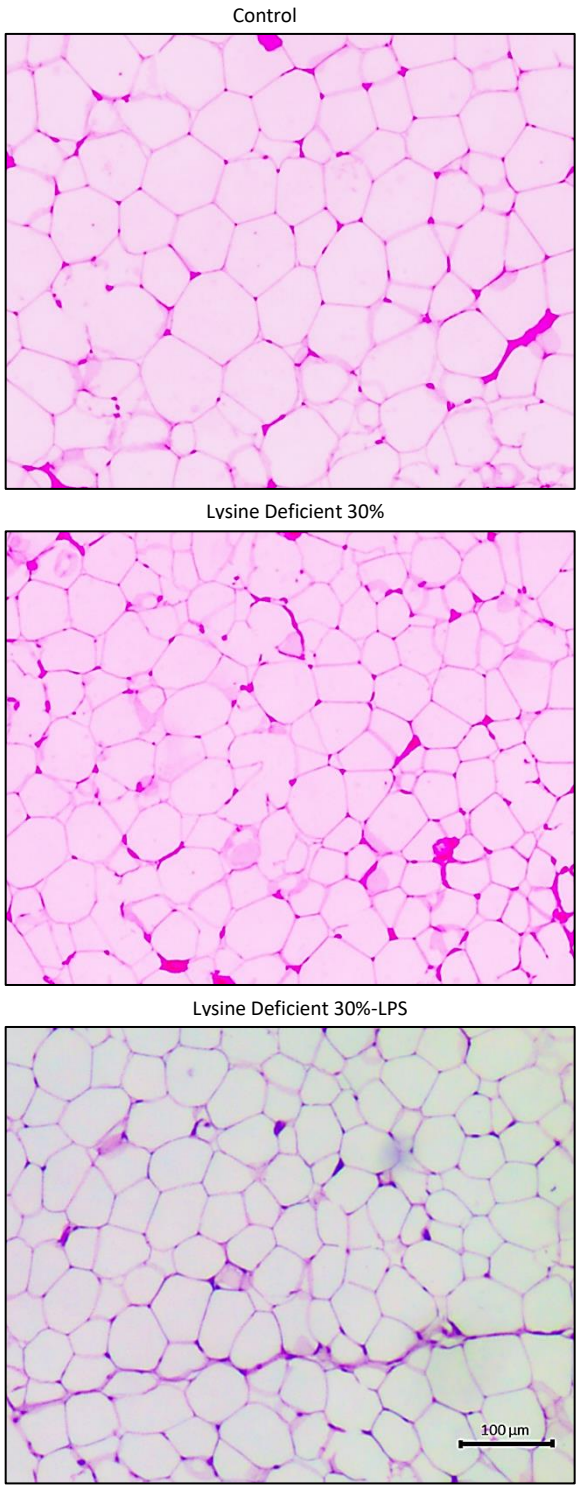

Figure 35. Representative H&E staining of epididymal fat pad from the different groups of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

Hematoxylin and Eosin (H&E) staining was done on sections of the epidydimal fat pad of rats fed a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge. Images were taken at 4x magnification under a light microscope. Scale bar represents 100 µm.

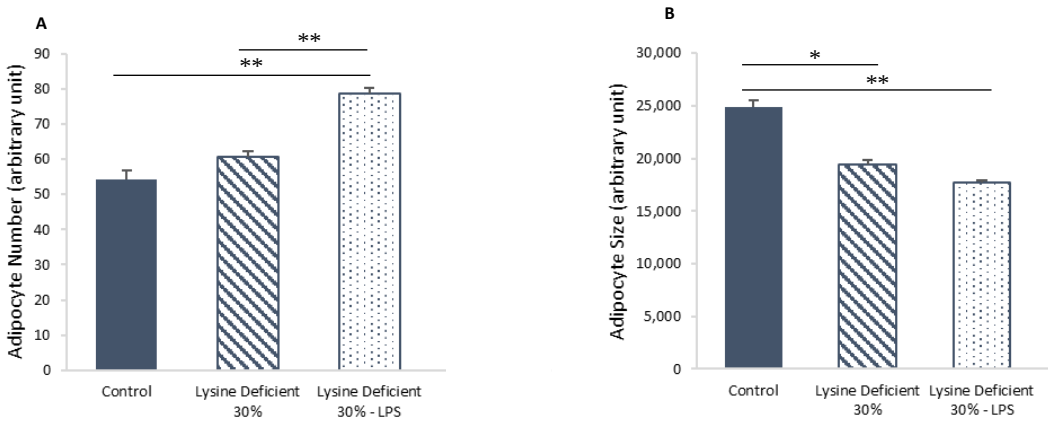

Figure 36. Epididymal adipocyte number and size in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

Hematoxylin and Eosin (H&E) staining was done on sections of the epidydimal fat pad of rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=5) or absence of LPS challenge (n=5). Images were taken at 4x magnification under a light microscope per group). The size of adipocytes was estimated using ImageJ software, and quantification was done manually for a set area in all slides. Data are expressed as mean±SEM and were analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01).

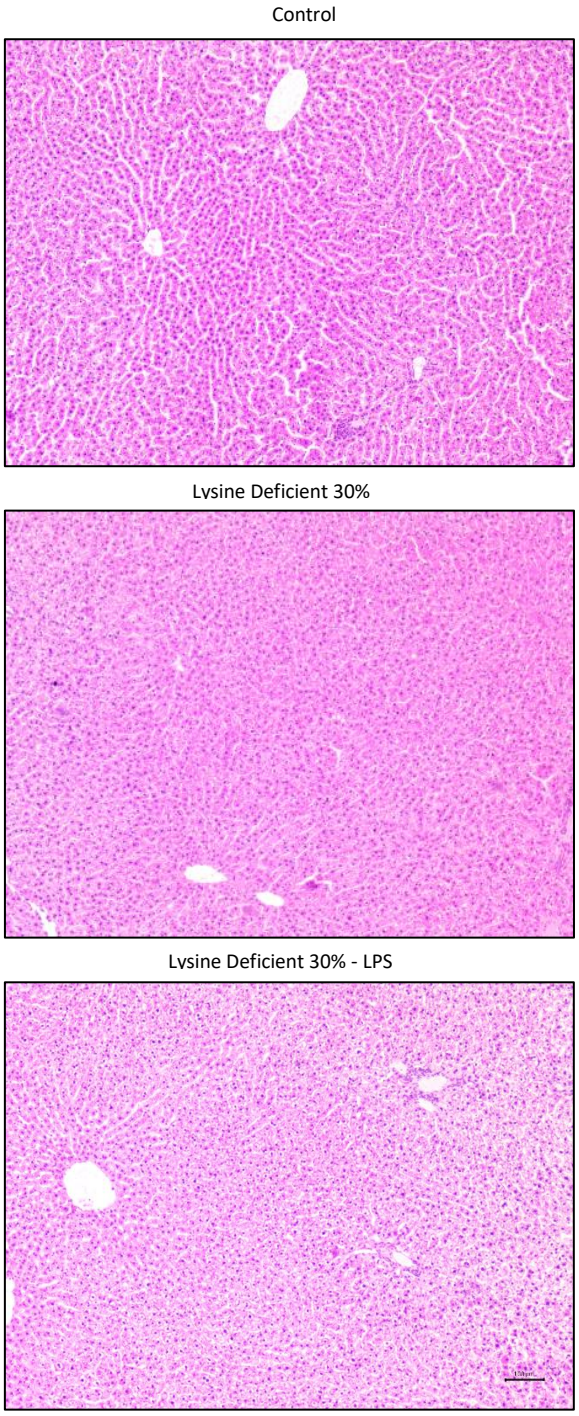

Figure 37. Representative H&E staining of liver sections from the different groups of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

Histopathological appearance of liver sections stained with Hematoxylin and Eosin at 4x magnification power in rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=5) or absence of LPS challenge (n=5). Scale bar represents 100 µm.

3. Blood

a. Glycemic

No difference between groups was seen for glycemia (*p*-value=0.103), insulin (*p*-value=0.100), GLP-1 (*p*-value=0.163), and HOMA-IR (*p*-value=0.152). The GLM analysis revealed a slight yet significant decrease in glycemia attributed to lysine deficiency (*p*-value=0.044). However, no other effects were described (Figure 38).

b. Lipid profile and pH-mediated parameters

One-way ANOVA showed that lipid profile was similar between groups. Nevertheless, when performing a GLM analysis, lysine deficiency did not affect lipoprotein levels, but LPS administration increased total cholesterol (*p*-value=0.043) by increasing HDL (*p*-value=0.032) (Table 12).

Besides, no differences were detected in sodium, chloride, and lactate (Table 12).

c. Vasopressin

Vasopressin was not significant between groups (*p*-value=0.531), and no effects were seen for lysine deficiency or LPS (Figure 39).

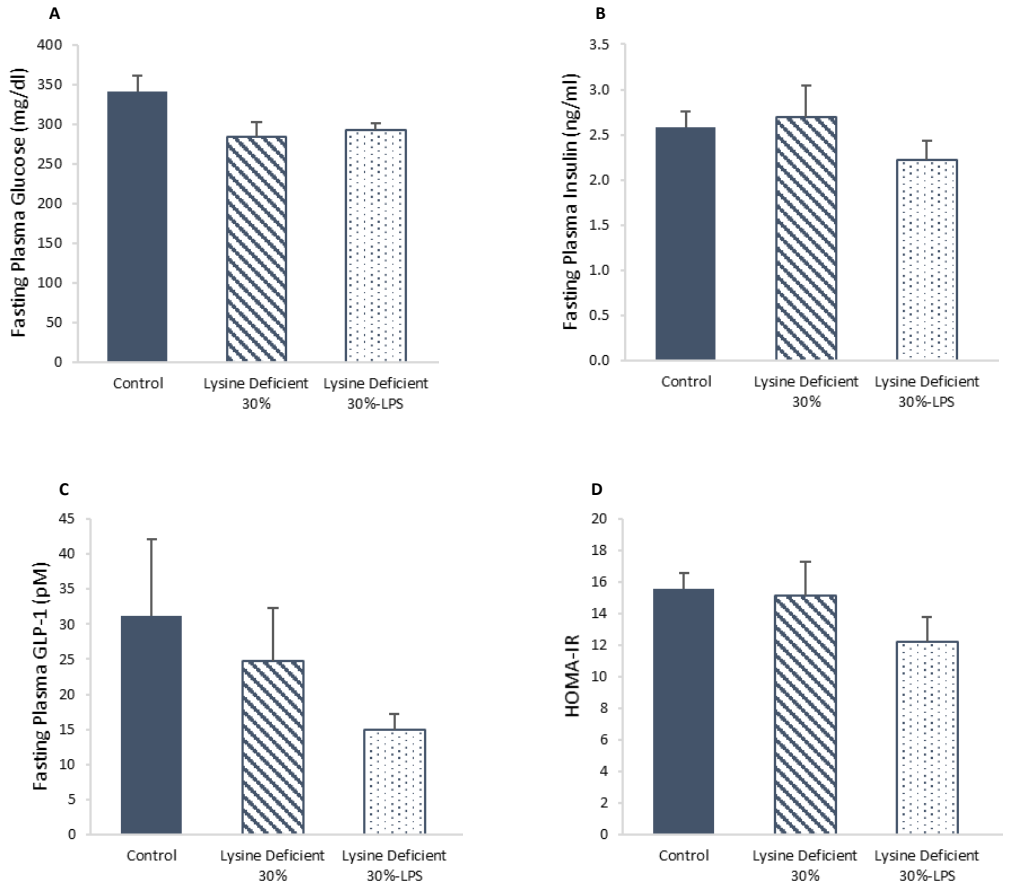

Figure 38. Glycemic profile of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) Fasting plasma glucose, (B) Fasting plasma insulin, (C) Fasting plasma GLP-1, and (D) HOMA-IR index were measured in rats fed a control diet (n=4) or a 30% lysine deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05

Table 12. Fasting plasma lipid profile and pH-medicated parameters of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

	Control	Lysine Deficient 30%	Lysine Deficient 30%-LPS	ANOVA *p*-value
Lipid profile				
Triglyceride (mg/dl)	107.0±44.6	77.6±17.3	69.0±20.7	0.086
Total Cholesterol (mg/dl)	82.8±8.3	86.3±8.2	97.5±12.6	0.160
HDL (mg/dl)	57.0±4.5	59.8±5.8	67.3±12.6	0.088
LDL (mg/dl)	19.1±6.7	14.2±7.7	12.3±8.5	0.528
pH-mediated parameters				
Sodium (mmol/L)	138.5±1.3	138.6±1.2	139.3±0.7	0.173
Chloride (mmol/L)	91.0±0.8	91.3±0.8	91.3±1.5	0.884
Lactate (mmol/L)	7.3±0.7	6.1±0.9	6.9±0.8	0.065

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Parameters were assessed in rats fed a control diet (n=4) or a 30% lysine deficient diet in the presence (n=8) or absence of LPS challenge (n=8).
Statistical significance was set at p-value<0.05.

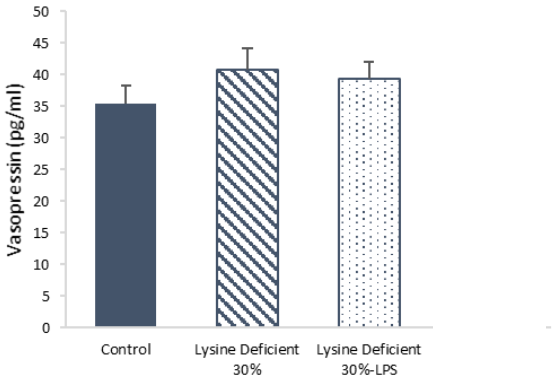

Figure 39. Plasma vasopressin levels in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

Plasma vasopressin levels of rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=6) or absence of LPS challenge (n=6).

Data are expressed as the mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05.

d. Amino acid

Free amino acids presented at varied concentrations in the groups (Table 13). Interestingly, lysine deficiency dropped plasma lysine concentration by around 36%, and this was the only impact observed for lysine deficiency. The other significant variations for histidine, leucine, alanine, citrulline, glycine, and serine were explained by the increasing effect of LPS. Despite some single amino acid variations, the total count for essential and non-essential amino acids was similar among groups, which resulted in comparable overall concentrations. Representative chromatograms are presented in Figure 40.

Table 13. Plasma amino acid levels of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

Amino Acids (µmoles/L)	Control	Lysine Deficient 30%	Lysine Deficient 30%-LPS	*ANOVA* *p*-value
Essential amino acids				
Histidine	78.3±11.5[ab]	68.1±6.0[a]	101.5±29.2[b]	**0.044**
Isoleucine	87.0±18.5	99.1±14.5	105.7±23.2	0.292
Leucine	169.3±27.0[ab]	170.7±25.9[a]	217.6±41.9[b]	**0.032**
Lysine	470.8±118.2[a]	300.9±84.6[b]	297.3±34.1[b]	**0.006**
Methionine	50.3±3.2	50.9±6.0	60.9±14.7	0.324
Phenylalanine	82.0±6.1	73.1±6.5	86.9±17.1	0.130
Threonine	218.5±31.0	190.1±22.5	225.9±39.7	0.071
Tryptophan	75.0±6.6	73.9±13.6	90.4±23.2	0.369
Valine	175.0±5.3	176.8±22.1	215.0±53.2	0.087
Total	1268.3±191.1	1143.4±204.1	1344.1±204.3	0.132
Non-essential amino acids				
Alanine	624.5±107.5[a]	550.4±77.6[a]	766.6±84.8[b]	**0.001**
Arginine	6.0±3.6	20.8±11.8	27.6±18.9	0.112
Asparagine	46.5±5.8	44.1±15.9	50.0±13.7	0.368
Citrulline	80.0±6.0[a]	98.5±8.5[a]	138.0±21.6[b]	**0.001**
Glutamic acid	308.3±66.5	301.6±66.2	361.0±72.9	0.194
Glutamine	714.0±86.4	691.3±141.9	877.4±171.4	0.063
Glycine	362.0±95.3[ab]	345.7±65.6[a]	502.7±102.1[b]	**0.032**
Serine	407.5±71.9[a]	405.5±57.6[a]	512.6±65.6[b]	**0.037**
Taurine	349.0±158.0	236.9±72.2	320.7±55.2	0.214
Tyrosine	121.5±21.7	97.4±16.2	105.2±12.5	0.084
Ornithine	247.0±30.4	216.4±34.9	267.1±62.5	0.284
Total	3006.0±332.3	2960.1±393.1	3513.8±677.6	0.114
Overall Total	4275.3±477.3	4104.5±575.6	4858.9±828.1	0.100

Data are expressed as the mean±SD and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Amino acids were assessed in rats fed a control diet (n=4) or a 30% lysine deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Means with the same letter are not significantly different (p-value>0.05).

Statistical significance was set at p-value<0.05.

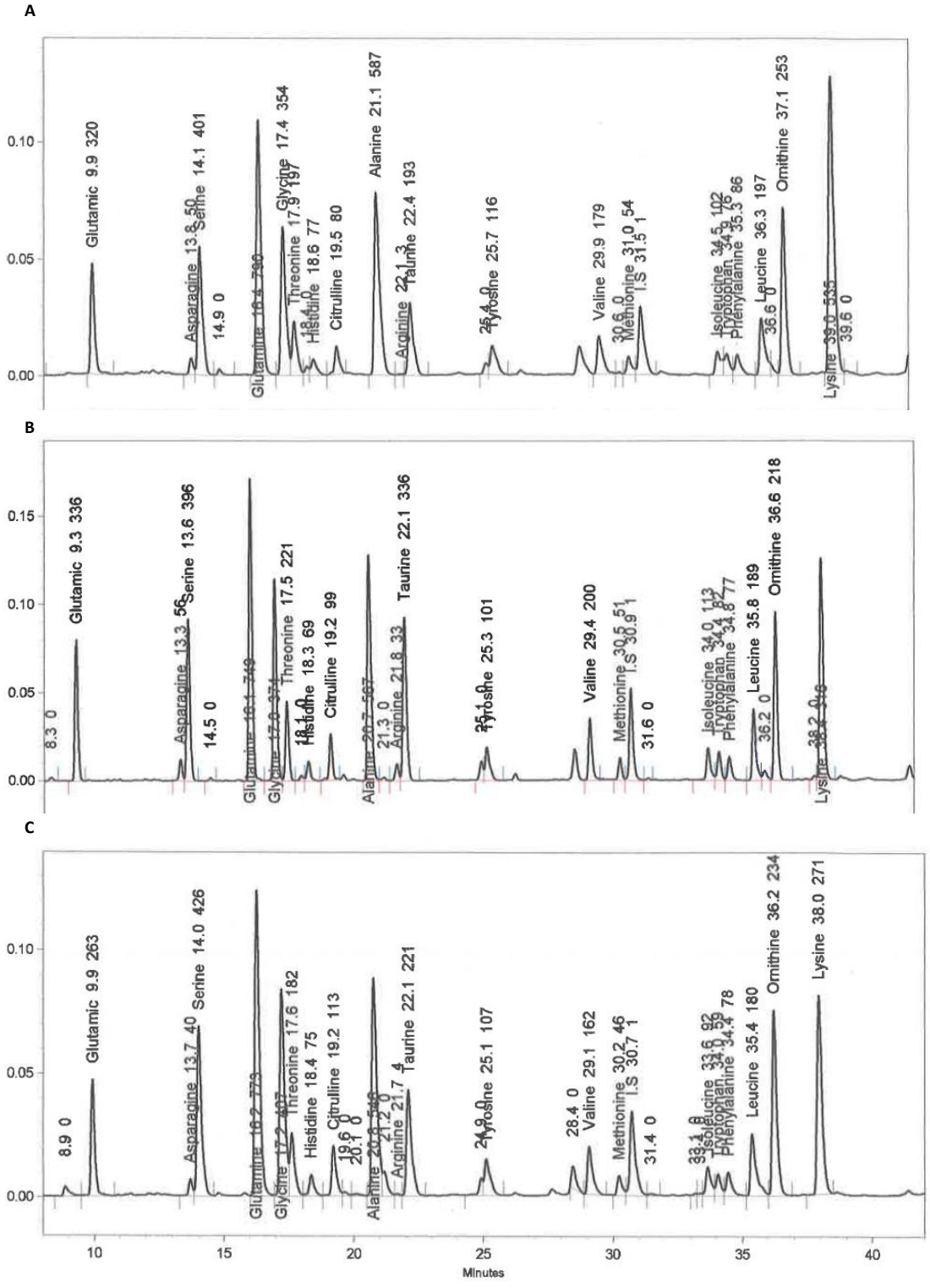

Figure 40. HPLC chromatograms of plasma amino acids in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

A sample amino acid chromatography in plasma of the (A) Control, (B) Lysine Deficient 30%, (C) Lysine Deficient 30%-LPS group.

4. Albumin

As shown in Figure 41, qRT-PCR indicated no difference in albumin mRNA expression in the liver (*p*-value=0.512).

According to one-way ANOVA, plasma albumin concentration was not different in the three groups (*p*-value=0.134). However, the GLM analysis revealed a small yet significant decreasing effect of lysine deficiency (*p*-value=0.034) but not LPS (*p*-value=0.189) (Figure 41).

5. CRP

mRNA and protein expressions of CRP in the liver did not change (*p*-value=0.750 and 0.205, respectively). The GLM analysis described a tendency of increase in the hepatic protein expression with lysine deficiency (*p*-value=0.080) but not with LPS (*p*-value=0.263) (Figure 42).

Results of the epididymal fat pad displayed a rise in the mRNA expression of CRP associated with LPS (*p*-value=0.025), yet this finding was not supported by the protein expression (*p*-value=0.841) (Figure 43).

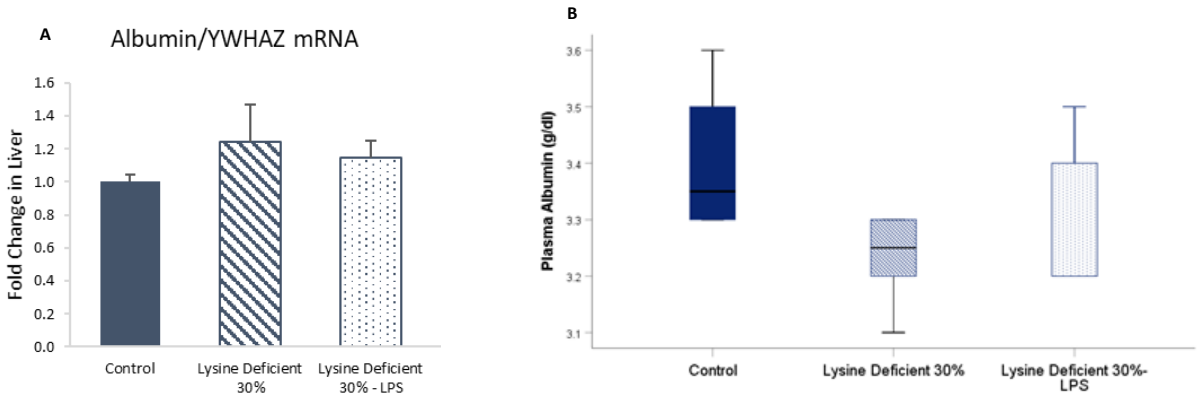

Figure 41. mRNA expression and plasma levels of albumin in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) qRTPCR result showing the relative mRNA expression of albumin in the liver (n=4 for Control and n=5 for Lysine Deficient and Lysine Deficient-LPS) and (B) Fasting plasma albumin levels (n=4 for Control and n=8 for Lysine Deficient and Lysine Deficient-LPS) of rats fed a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge.

mRNA data are expressed as mean±SEM and plasma levels are expressed as median (the bar inside the box), the interquartile range (the box length), the minimum value (the lower bar cap) and the maximum value (the upper bar cap). Results were analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Boxes of groups sharing the same letter have statistically similar results.

Statistical significance was set at p-value<0.05.

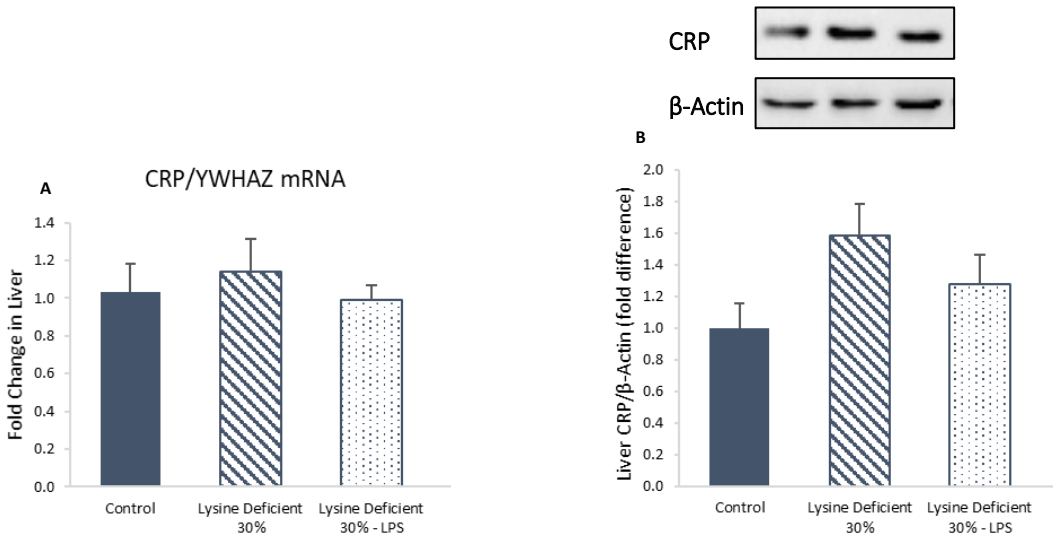

Figure 42. Hepatic mRNA and protein expressions of CRP in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) qRTPCR result showing the relative mRNA expression of CRP (n=4 for Control and n=5 for Lysine Deficient 30% and Lysine Deficient 30%-LPS) and (B) Western Blot result presenting protein CRP expression (n=4 for Control and n=8 for Lysine Deficient 30% and Lysine Deficient 30%-LPS) in the liver of rats fed a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge. Protein bands detected by Western blots were quantified with Image J software and normalized against β-Actin. Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05.

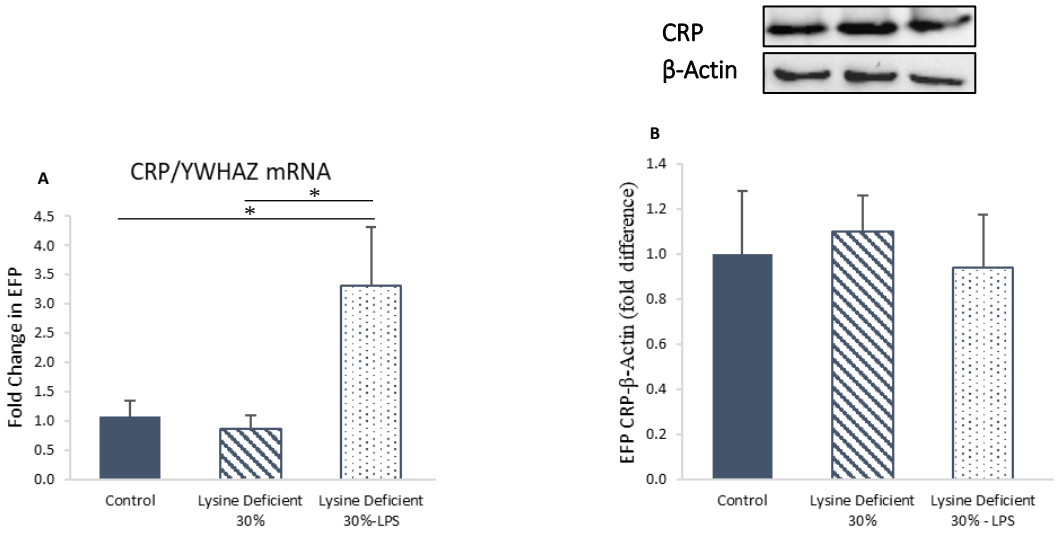

Figure 43. Epididymal fat pad mRNA and protein expressions of CRP in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

EFP: Epididymal fat pad

(A) qRTPCR result showing the relative mRNA expression of CRP (n=4 for Control and n=5 for Lysine Deficient and Lysine Deficient-LPS) and (B) Western Blot result presenting protein CRP expression (n=4 for Control and n=8 for Lysine Deficient and Lysine Deficient-LPS) in the liver of rats fed a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge.

Protein bands detected by Western blots were quantified with Image J software and normalized against β-Actin. Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05 (* p-value<0.05).

6. Other inflammatory markers

The qRT-PCR showed different mRNA expressions of inflammatory markers among groups. In the liver, TLR4 (*p*-value=0.683), MyD88 (*p*-value=0.940), and IL6-R (*p*-value=0.261) expressions were very similar among the groups. However, IL-1β mRNA expression was increased with LPS (*p*-value=0.002) (Figure 44), and IL-6, TNF-α, and INF-γ were not expressed in the liver.

LPS was shown to stimulate the expression of TLR4 (*p*-value=0.006), MYD88 (*p*-value= 0.043), and IL-1β (*p*-value=0.005) in the epididymal fat pad (Figure 45). Though, IL-6, IL-6R, TNF-α, and INF-γ were not expressed.

At the level of the spleen, only MyD88 (*p*-value=0.043) and IL-6 (*p*-value<0.001) showed a significantly increased expression with LPS administration, while other parameters (TLR4, IL-6R, IL-1β, and INF-γ) only expressed a trend (Figure 46). TNF-α was not detected in the spleens of these groups.

No effect of lysine deficiency was identified at any level.

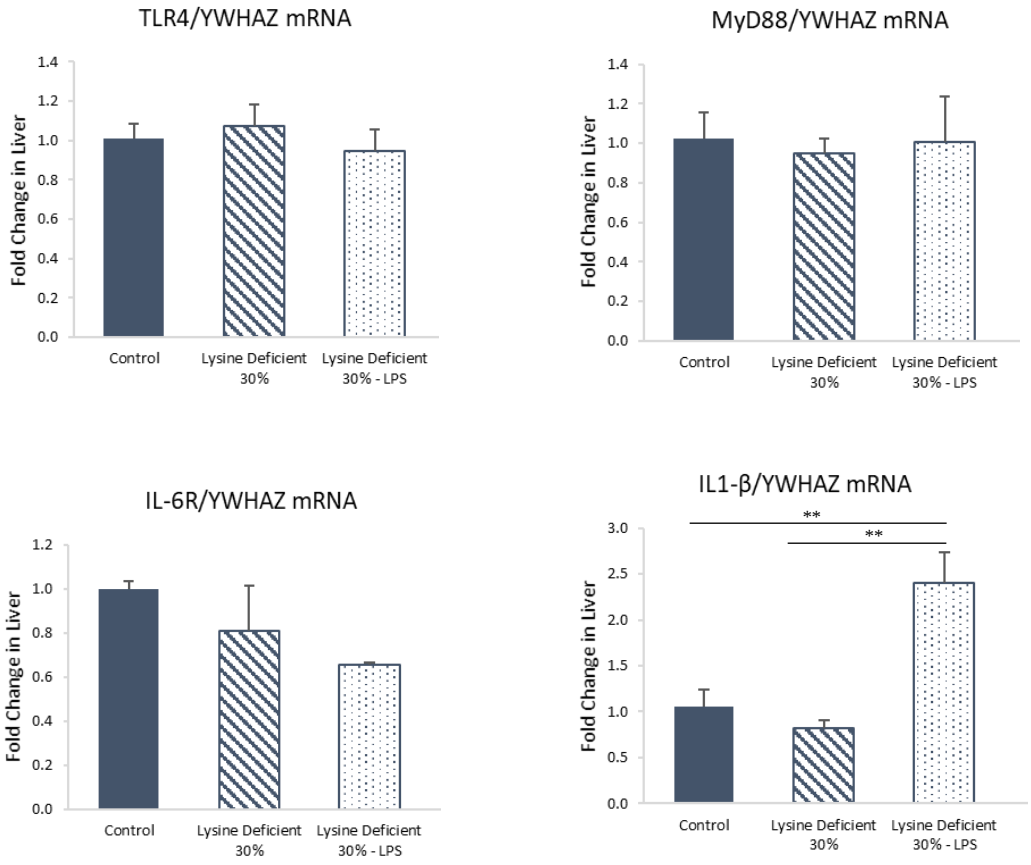

Figure 44. mRNA expression of TLR4, MyD88, IL-6R, and IL-1β in the liver of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

qRT-PCR results showing the relative mRNA expression of TLR4, MyD88, IL-6R, and IL-1β in the liver of rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=5) or absence of LPS challenge (n=5).

Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (** p-value<0.01).

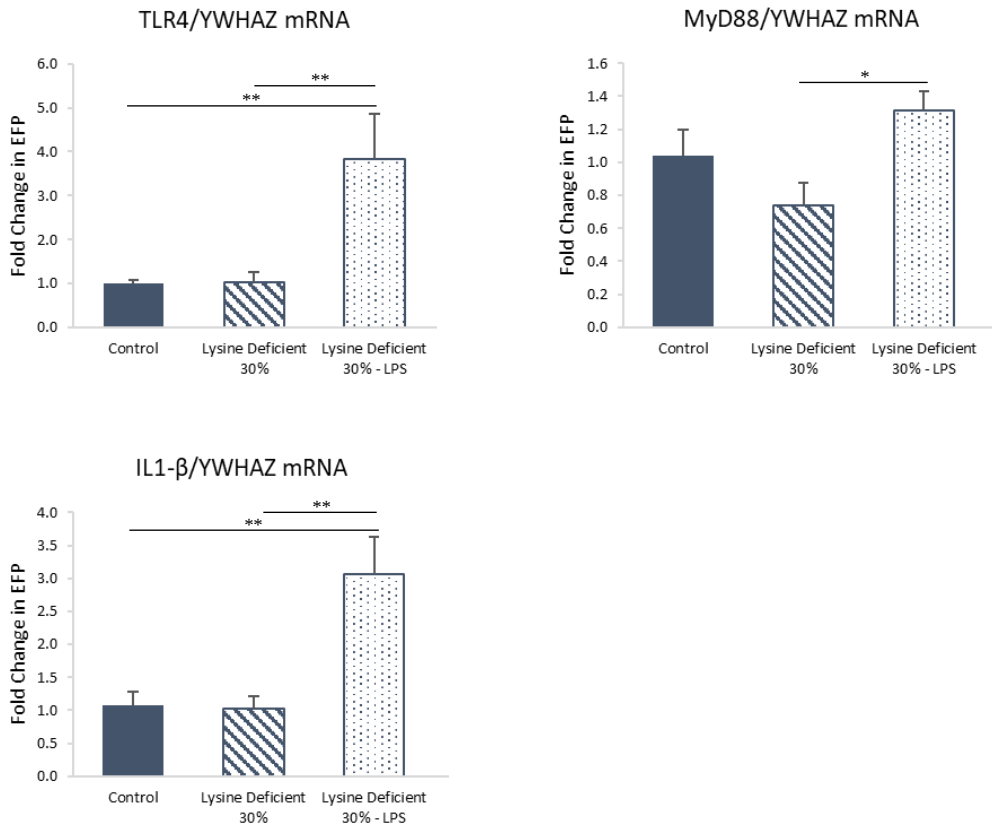

Figure 45. mRNA expression of TLR4, MyD88, and IL-1β in the epididymal fat pad of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

EFP: Epididymal fat pad.

qRT-PCR results showing the relative mRNA expression of TLR4, MyD88, and IL-1β in the epididymal fat pad of rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=5) or absence of LPS challenge (n=5).

Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (*p-value<0.05, ** p-value<0.01).

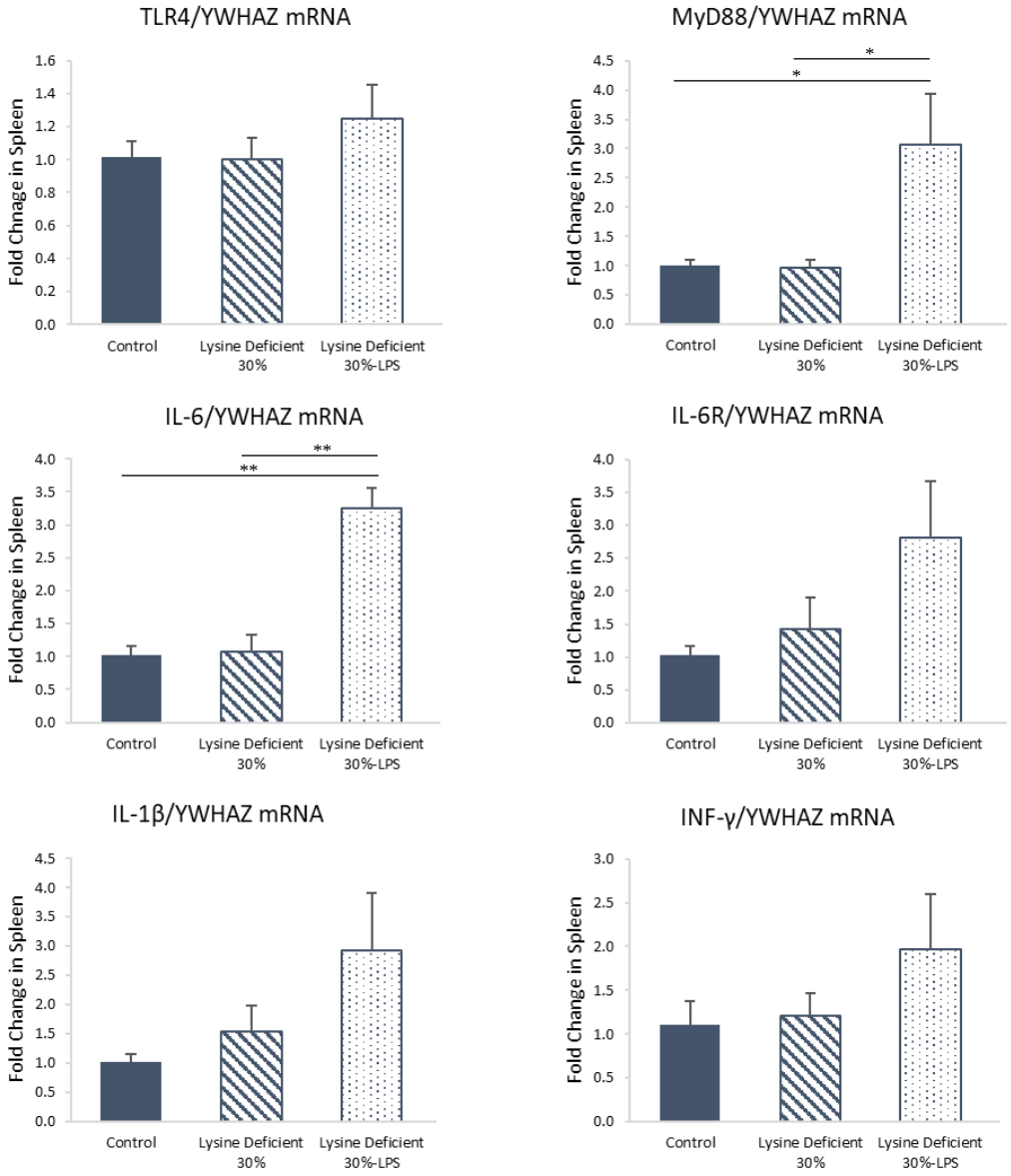

Figure 46. mRNA expression of TLR4, MyD88, IL-6, IL-6R, IL-1β, and INF-γ in the spleen of rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

qRT-PCR results showing the relative mRNA expression of TLR4, MyD88, IL-6, IL-6R, IL-1β, and INF-γ in the spleen of rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=5) or absence of LPS challenge (n=5).

Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05 (*p-value<0.05, ** p-value<0.01).

7. Behavioral tests

Pain test was performed during the last week before euthanasia, and differences were not detected among groups (*p*-value=0.256) (Figure 47).

The OFT showed no discrepancies in sessions, groups, or their interaction when two-way repeated measure ANOVA was performed (Figure 48). This result was also supported using one-way ANOVA on parameters in separate sessions. The total distance traveled, distance traveled in the peripheral area, and the time spent in corners were similar between groups in session 1, session 2, and session 3 (Tables 49-51). In line with the lack of significance, the distance traveled in other arenas, the percentage of distance and time in the three different arenas (peripheral, internal, and central) were all similar among the groups (*p*-value>0.05).

Sample trajectories of the experimental groups in session 1 are illustrated in Figure 52.

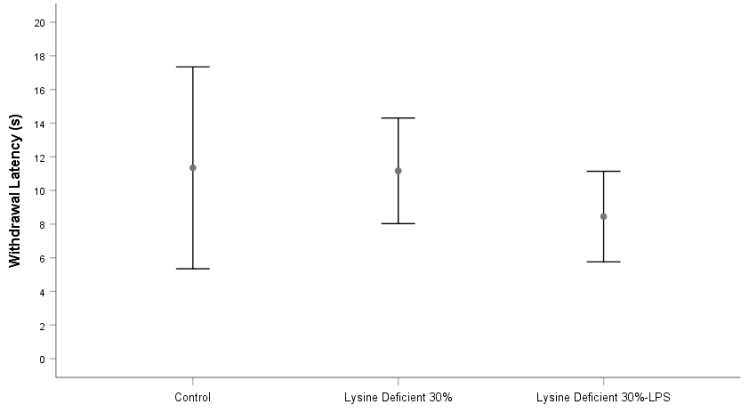

Figure 47. Thermal pain sensation in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

This heat stimulus was applied thrice per rat, separated by an interval of 5 minutes. The average of the two closest values was reported. Data are expressed as mean±SEM and analyzed by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05.

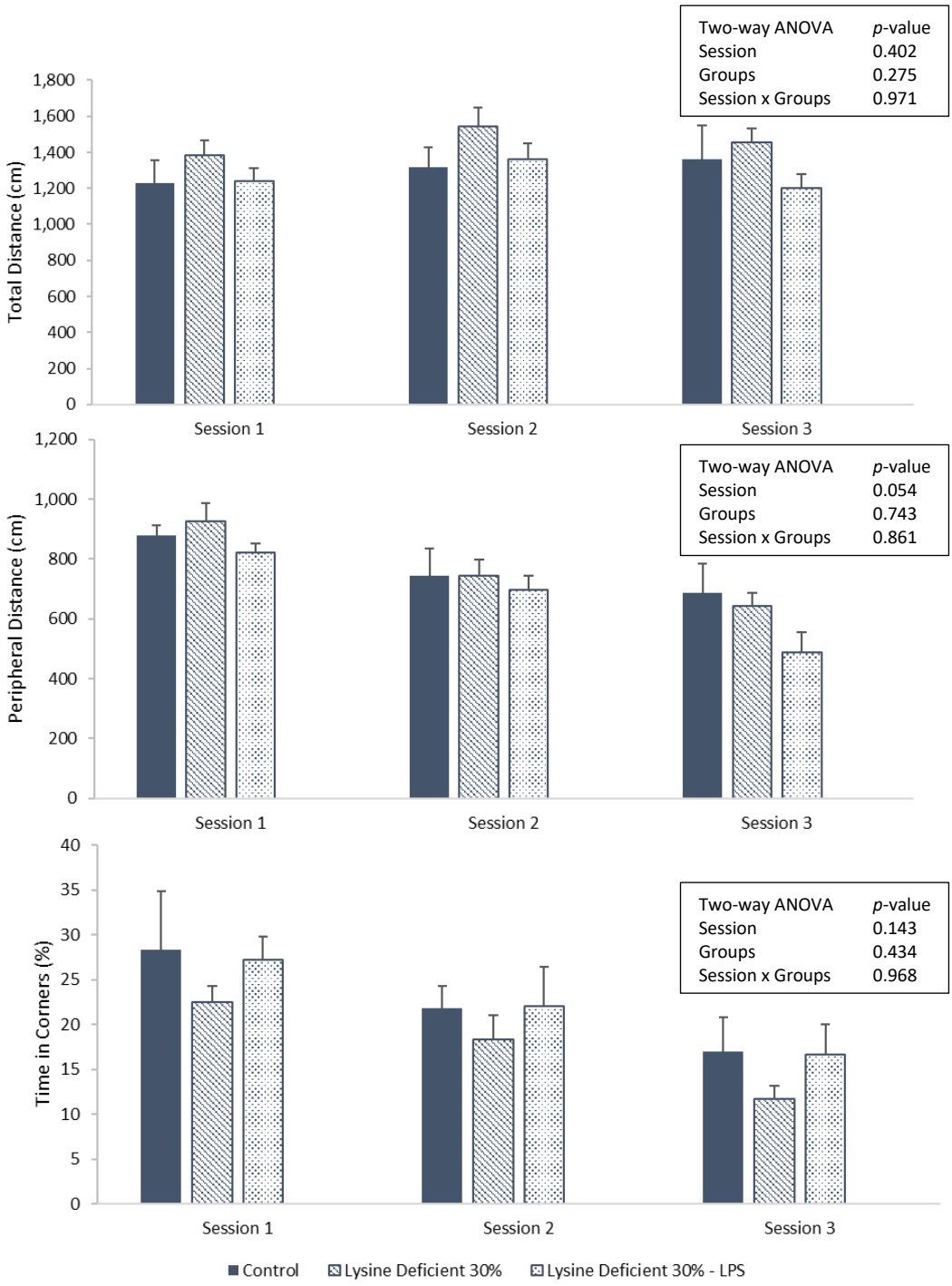

Figure 48. Total distance, distance in the periphery, and time spent in corners over the three sessions of the Open Field Test in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) Total distance traveled over the three consecutive sessions, (B) Distance traveled in the peripheral area over the three consecutive sessions, and (C) The percent time spent in corners over the three consecutive sessions by rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as mean±SEM and described using SMART software. Analysis was done by a two-way repeated measures ANOVA to detect variation of means in groups over repeated sessions among groups followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons. Statistical significance was set at p-value<0.05.

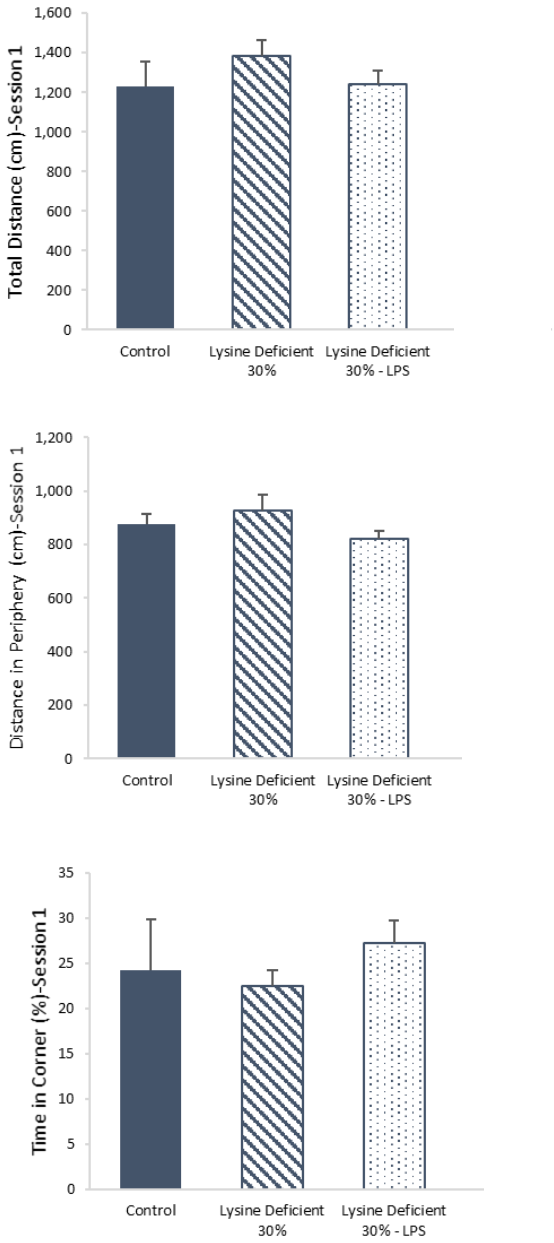

Figure 49. Total distance, distance in the periphery, and time spent in corners in session 1 of the Open Field Test in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) Total distance traveled, (B) Distance traveled in the peripheral area, and (C) The percent time spent in corners, during session 1, by rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as mean±SEM and described using SMART software. Analysis was done by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05 (* p-value<0.05; ** p-value<0.01)

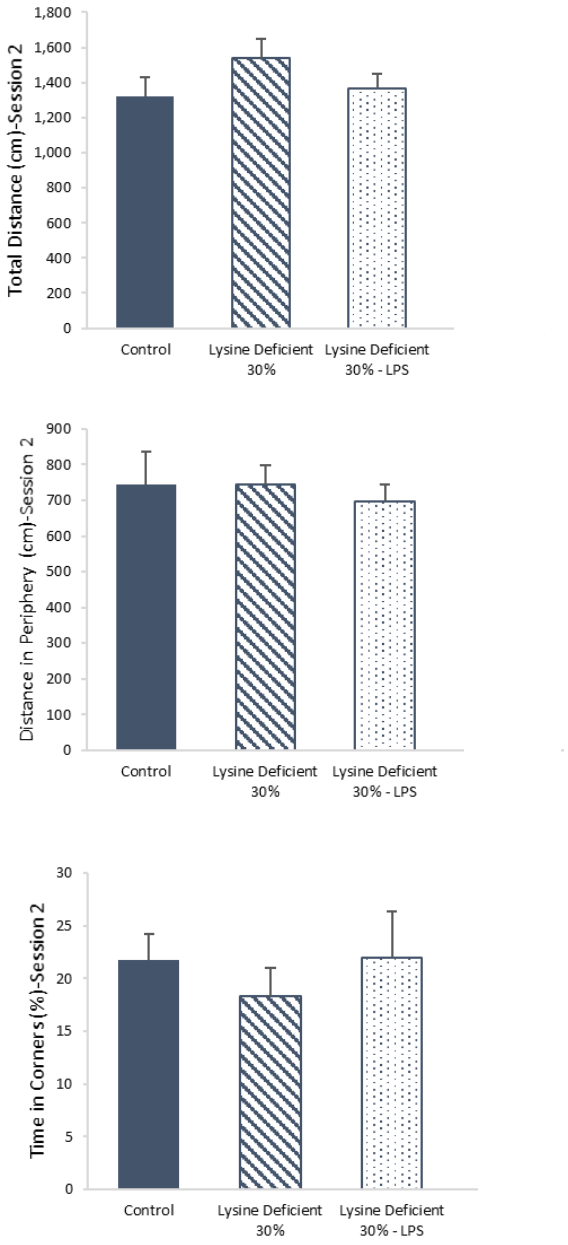

Figure 50. Total distance, distance in the periphery, and time spent in corners in session 2 of the Open Field Test in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) Total distance traveled, (B) Distance traveled in the peripheral area, and (C) The percent time spent in corners, during session 1, by rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as mean±SEM and described using SMART software. Analysis was done by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05

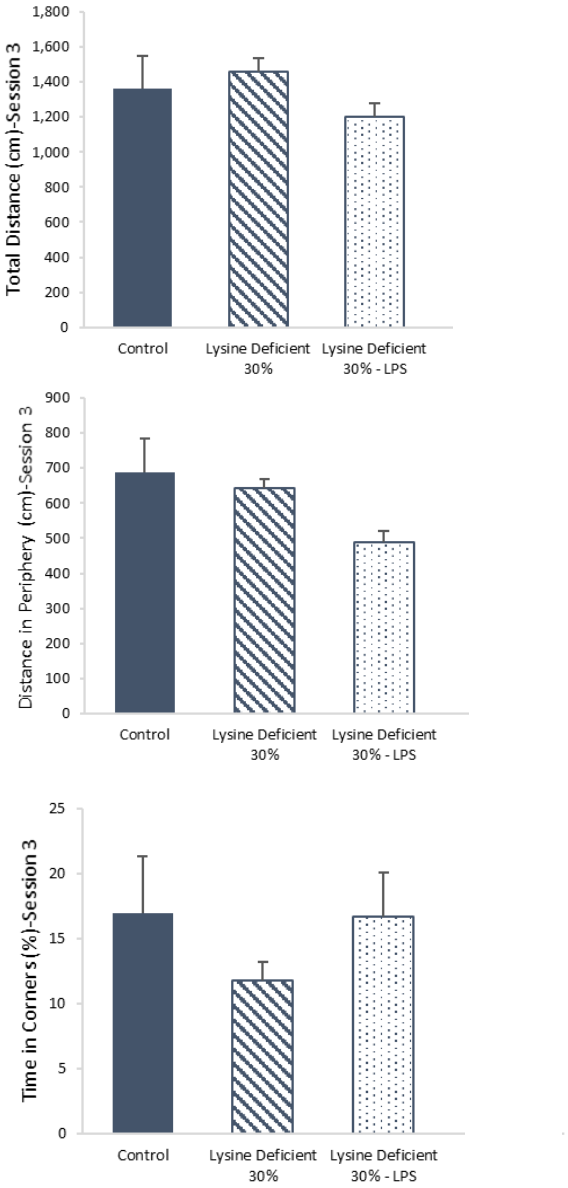

Figure 51. Total distance, distance in the periphery, and time spent in corners in session 3 of the Open Field Test in rats maintained on a control diet or a 30% lysine-deficient diet in the presence or absence of LPS challenge

(A) Total distance traveled, (B) Distance traveled in the peripheral area, and (C) The percent time spent in corners, during session 1, by rats fed a control diet (n=4) or a 30% lysine-deficient diet in the presence (n=8) or absence of LPS challenge (n=8).

Data are expressed as mean±SEM and described using SMART software. Analysis was done by one-way ANOVA to detect the difference between groups, followed by Fisher's least significant difference (LSD) for post hoc multiple comparisons.

Statistical significance was set at p-value<0.05

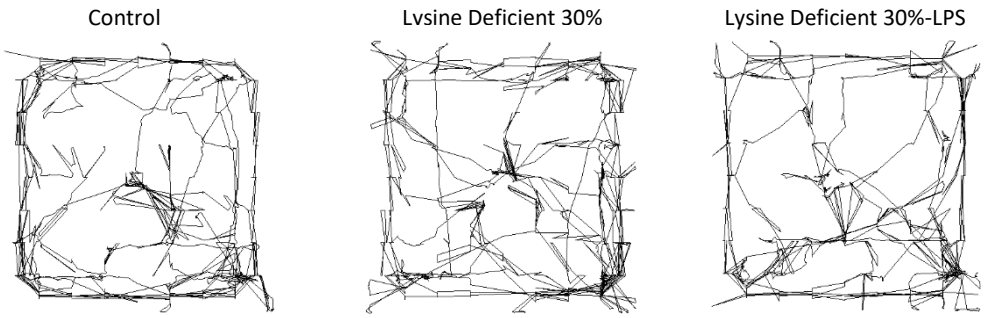

Figure 52. Sample trajectory of the group during session 1 of the Open Field Test
The trajectories were recorded for 5 minutes via a video camera connected to the tracking software Smart.

C. Relationship between lysine (intake and plasma concentration) and albumin

We were able to show in this work that a linear relationship exists between lysine intake on the one hand and plasma lysine and albumin concentration on the other (Figure 52). Pearson correlation was performed on pooled data from both experiments, and the results were highly significant. Strong positive correlations exist between lysine intake and plasma lysine levels ($r=0.758$; p-value<0.001) and lysine intake and albumin levels ($r=0.836$; p-value<0.001). Graphical representation of the association showed a parallel trend (Figure 52).

In addition, a significant positive linear relationship connects albumin and lysine plasma concentrations ($r=0.604$; p-value<0.001), as illustrated in Figure 53.

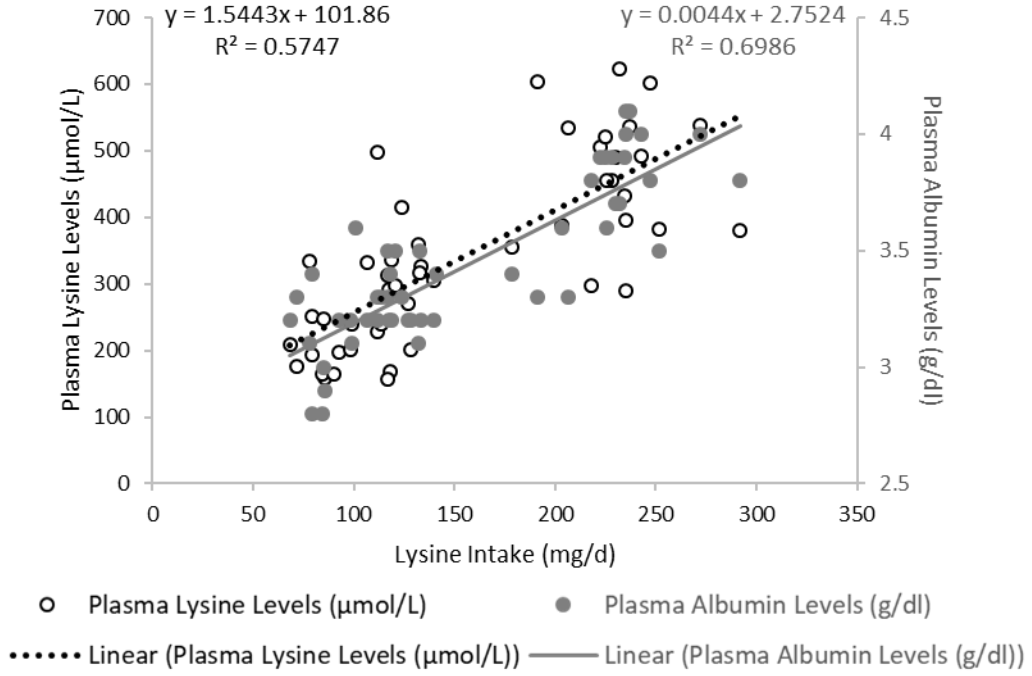

Figure 53. Correlation between lysine intake and plasma levels of lysine and albumin
Data from both experiments were pooled and plotted to visualize the association between lysine intake and plasma concentrations of albumin and lysine. Lysine intake was computed based on food intake of the rats and the lysine content of the corresponding experimental diet.

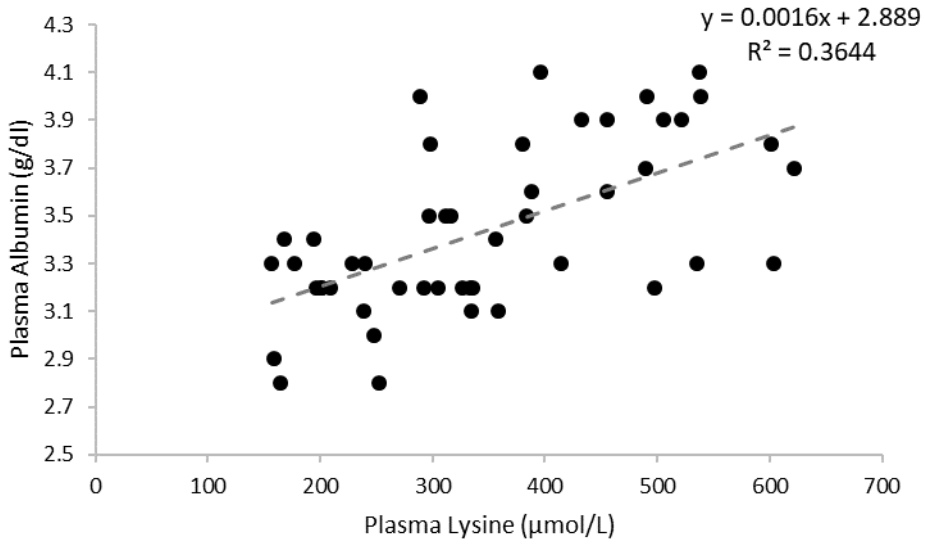

Figure 54. Correlation between plasma levels of lysine and albumin
Data from both experiments were pooled and plotted to visualize the association between plasma concentration of albumin in relationship to the one of lysine.

CHAPTER V

DISCUSSION

In this study, we hypothesized that lysine deficiency mediates an inflammatory profile and a perturbation in behavior in addition to the conventional phenotype of amino acid deficiency at the body weight and compartment levels. The observed modifications were irrespective of any simultaneous inflammatory trigger. Herein we demonstrated that the intake of 40% of the recommended amount of lysine in rats was sufficient to diminish growth and alter body composition in a parallel manner. Most importantly, 60% lysine deficiency induced a decrease in albumin concentration and stimulated hepatic CRP expression, which seems to be independent of the classical CRP production pathway. Notably, 60% lysine deficiency promoted hyperactivity and anxiety-like behavior, demonstrating the involvement of lysine in normal physiology at all levels. The presence of LPS exacerbated outcomes related to body composition, metabolism, and behavior caused by 60% lysine deficiency but did not worsen albumin and CRP expressions.

Interestingly, in this work, the deficiency of 30% presented a milder form of alteration, hinting at the existence of dose-dependent defects attributed to lysine deficiency.

Lysine is an essential amino acid that is not internally produced and does not participate in transamination reactions (Bhagavan & Ha, 2011), hence the importance of providing it in the diet. The role of lysine as a building block for protein synthesis is very supported in the literature. It serves as a precursor of the signaling pathway

for the mammalian target of rapamycin (mTOR), which is involved in protein synthesis. Lysine promotes muscle satellite cells proliferation and reduces their apoptosis through the mTORC1 signaling pathway (Jin et al., 2019; Liu et al., 2022). Also, it can inhibit the autophagic-lysosomal system of the myofibrillar proteins through the phosphorylation of AKT. Lysine restriction in the diet was shown to suppress growth and development that mostly affected muscle and lean mass (Kligler & Krehl, 1950; Ragi et al., 2019; Tian et al., 2019). The loss in muscle mass during lysine restriction was confirmed by an increase in the fractional rate of proteolysis (Tesseraud et al., 1996). Indeed, in our study, the impact on lean mass was proportional to the level of deficiency, with a difference to control of 100 g in 60% deficient group and 20 g in 30% deficient group at week 3.

While the amount of lysine in diet contributed to the main anthropometric differences, LPS has also conferred a minor yet significant effect in decreasing weight, fat, and lean masses. It has been shown that in case of any immune trigger (LPS administration, for example), amino acid repartitioning takes place, favoring the activation of the immune system and compromising protein synthesis related to growth (Kampman-van et al., 2016). In addition, LPS administration yields a significant increase in tissue and blood pro-inflammatory cytokines concentrations, which was indeed the case in our study. Among groups with LPS, gene expressions of IL-6, IL-1β, and/or TNF-α, in some organs were higher than the unchallenged groups. These markers can infiltrate the thyroid gland, reduce thyroxine and triiodothyronine production, and consequently blunt growth and development (Campos et al., 2014). In addition, immune reactions are known to increase lipolysis, resulting in a decrease in fat mass and an upsurge of free fatty acid in the circulation. This phenomenon can happen

through the binding of LPS (via its lipid A moiety) to its receptor, the toll-like receptor 4 (TLR4), stimulating its downstream inflammatory pathway (Zu et al., 2009). Indeed, gene expression of TLR4 and/or its downstream elements (MyD88 and cytokines) increased in selective tissues among stimulated rats. This fact feeds into the lipolytic hypothesis associated with LPS and explains the significant decrease in total body fat accretion, epididymal adipose tissue weights, and size of adipocytes. Besides, since fasting insulin levels were similar between LPS-administered and control groups, it is reasonable also to postulate the presence of an insulin-independent lipolytic route. Independently from changes in insulin, when inositol-requiring protein 1 (IRE1), a component of the endoplasmic reticulum stress, is activated in the adipocytes due to inflammation, it induces lipolysis (Foley et al., 2021). The lipolytic cycles, feeding the circulation with free fatty acids, further stimulate the inflammatory outcomes through the binding of free fatty acid with TLR4, dragging the system into a vicious cycle (Shi et al., 2006).

The difference in body weight and composition was not related to the total energy intake but to energy efficiency. Our data showed that rats with both deficiency levels had comparable feed and energy intakes to the control groups. Nevertheless, findings in the literature regarding food intake were inconsistent and can be explained by the variations in the level of deficiencies, age of the animals, etc. For instance, a study on pigs reported an increase in feed intake with 30% lysine limitation in baby animals but not in growing and adult pigs (Yin et al., 2018). Along with the same level of lysine insufficiency, piglets also increased feed intake (Yin et al., 2017). On the contrary, rats maintained on unsupplemented gluten diets (equivalent to 60% deficiency) diminished their food intake (Ragi et al., 2019). Similar total levels of

circulating amino acids might have contributed to comparable feed and energy intake among the groups, in harmony with the hypothesis that affirms a relationship between serum amino acid concentration and appetite (Mellinkoff et al., 1997).

When insufficient amounts of essential amino acids are available for protein synthesis, more amino acids remain in the circulation. This leads to a reduction in the energy cost for nutrient utilization destined for growth and development, known as energy efficiency. As a protective mechanism against amino acid accumulation, the body starts oxidizing more amino acids, which results in an increase in energy expenditure, witnessed among 60% lysine-deficient groups. In line with our findings, rats and pigs on lysine-deficient diets had poor energy efficiency (Millet & Aluwé, 2014; Ragi et al., 2019; Yang et al., 2009). Hence, the hyperactive behavior of our 60% lysine-deficient rats increased their non-exercise activity thermogenesis (NEAT), which may have helped to get rid of the accumulated amino acids in the form of energy. It is worth mentioning that the increase in energy expenditure and amino acid oxidation following a low-quality-protein diet is not limited to lysine deficiency only (Kampman-van et al., 2016; Ragi et al., 2019; Xiao & Guo, 2022).

Food intake is known to decrease with LPS administration (Chaskiel et al., 2019; Hu et al., 2011; O'Reilly et al., 1988; Sachot et al., 2004), which was not the case in this work. In pigs, LPS at a concentration of 1.5 μg/kg intravenously (IV) reduced food intake compared to the saline group only 24 hour-post injections and this difference disappeared at 48 hours (Nordgreen et al., 2018). Thus, it could be that our rats decreased feed ingestion right after LPS administration and compensated in the following days. The fact that food intake was not measured on a daily basis did not allow us to confirm this hypothesis.

The hypodipsia associated with 60% lysine deficiency is probably related to lower body weight. The decrease in water intake did not seem to support homeostatic regulation as vasopressin levels were similar to control. A recent study on mice reported an increase in water intake through the stimulation of hepatic fibroblast growth factor 21 (FGF21) when either tryptophan or threonine but not lysine was restricted in the diet (Rusu et al., 2021).

Organs of the rats were different between groups and it is believed that both LPS administration and lysine deficiency have caused functional alterations. The enlargement of spleens in the presence of LPS shows an active immune status, which was in accordance with other studies (Liverani et al., 2014; Zhong et al., 2018). In well-nourished rats, the increase in weight triggered by LPS exceeds 50%, while in the deficient groups, the increase did not reach 40%, and the difference was statistically significant. This could be explained by a suboptimal immune response when an essential amino acid intake is inadequate.

Likewise, liver weights decreased with the magnitude of lysine deficiency, which was expected due to some metabolic limitations associated with insufficient amounts of lysine. The similarity in hepatic fat between groups implies that the increase in liver size with LPS is not attributed to an ectopic lipid accumulation but to the liver involvement in the body's immune response (Shao et al., 2011). In the current experiments, we failed to detect morphological changes in livers despite the functional and metabolic alterations and the increased weights.

The difference in gene expression in livers and spleens was dependent on the nutrition status (control diet vs. deficient diets), which implies that the body needs a minimal level of lysine to mount normal immune responses, under which the

immune reaction will be diminished (the case of 60% lysine deficiency). The only difference at the hepatic level was the expression of IL-1β for the LPS group, while the expression of most inflammatory markers was stimulated in the spleen. This clearly states that the liver does not mount a normal reaction to antigens in case of deficiency but prioritizes internal regulations. In fact, the liver and spleen convey pro-inflammatory cytokine expression at different rates; while hepatic responses are faster and shorter, the splenic ones are slower and more consistent (Floreste et al., 2023).

The weight of the epididymal fat pad of the rats confirmed the body composition findings; both lysine deficiency and LPS exhibited a decreasing effect. In addition, the strong positive correlation between total body fat and epididymal fat mass proves that variations in weight and fat mass reflect those in the visceral area. Also, the adipose tissue dynamics differed among the groups, with 60% and 30% lysine deficiency. For the same microscopic frame, the adipocytes were more numerous and/or smaller in size in the lysine-deficient diets compared to the control diet. These alterations pose serious cardiovascular implications (Pilz & März, 2008), presuming that this result is associated with higher levels of circulating free fatty acids.

Interestingly, lysine deficiency at different levels and/or our small dose of LPS did not affect the fasting glycemic profile. However, the IPGTT presented evidence of a lessened glycemic response associated with 60% lysine deficiency. It is worth noting that the glycemic profile is better depicted in the postprandial than in the fasting phase (Ketema & Kibret, 2015; Sheppard et al., 2005).

The observed reduction in total cholesterol in the 60% lysine-deficient groups was mainly related to a decrease in HDL rather than LDL fractions. The drop in the lipid profile (except for the LDL) is widely reported in the community among malnourished

populations (Akuyam et al., 2008; Rajesh et al., 2020). Because lysine is largely needed for the synthesis of these lipoproteins (Levy et al., 1967), a major drop in lysine administration is expected to yield a decrease in lipid profile. Reduced triglyceride of lysine deficient groups was not associated with an increase in HDL, in contrast to the commonly reported inverse relationship (Miller et al., 2007).

In addition, LPS did not cause changes in triglyceride levels with lysine deficiency, which was not the case with the control diet. In a well-nourished status, high levels of FFA induced by LPS are esterified to plasma triglycerides, an energy-requiring mechanism to reduce the cytotoxic effect of FFA (Foley et al., 2021).

Another remarkable finding caused by 60% and not 30% lysine deficiency was the increase in chloride levels. The reason behind the slight increase (~6%) in chloride concentration is not well understood, but it can indirectly contribute to the malnutrition cycle. When the pH of the medium decreases (due to higher chloride levels, for example), the total unbound FFA concentration increases, exacerbating the health status (Spector et al., 1969).

One of the major outcomes of our study was the effect of different levels of lysine deficiency on plasma amino acid concentrations. Plasma lysine concentration was positively correlated with lysine intake (R^2=0.5747), which makes plasma lysine concentration a good marker of lysine intake. Interestingly, the magnitude and type of alteration of other essential amino acids differed with the level of lysine deficiency. The majority of amino acids decreased with 60% lysine deficiency, and this is considered a hallmark associated with malnutrition (Polge et al., 1997). Unlike most amino acids, histidine and serine increased by around 50%, and threonine by 2.5 folds. The increase in threonine levels (and to a lower extent in histidine and

serine) in lysine deficiency was previously reported in the literature (Goda et al., 2021; Kim et al., 1996; Regmi et al., 2016; Roy et al., 2000; Zimmerman & Scott, 1965). This could be explained by a hindered utilization and/or oxidation of these amino acids. For instance, it is reasonable to assume a decrease in threonine utilization with 60% lysine deficiency. As previously discussed, 60% lysine deficiency induced a reduction in protein synthesis along with an increase in degradation, apoptosis, and autophagy. This scenario is not limited to skeletal muscles but can also be seen in mucosal proteins and others. A study on malnourished mice with induced anorexia typically illustrates the decrease in the colonic mucosal proteins during malnutrition (Nobis et al., 2018). Also, it is worth mentioning that the synthesis of mucosal proteins accounts for around 70% of total threonine use (Le Floc'h & Sève, 2005). Hence, threonine is expected to remain in the circulation when its principal use is drastically reduced. The impairment of mucin production with lysine deficiency probably results in a hindered absorption of nutrients, and this was reflected in the observed reduction of energy efficiency. Moreover, the increase in histidine did not have an anorectic effect, unlike what has been previously reported (Okusha et al., 2017). Histidine is converted to neuronal histamine in the hypothalamus (Yoshimatsu et al., 2002) and suppresses food intake by binding to histamine H1 receptor (Ookuma et al., 1989).

A mild lysine deficiency of 30% did not affect amino acid levels. Except for lysine, amino acid concentrations increased in this group, and this was attributed to LPS administration. This is consistent with a cross-sectional analysis among 19,472 subjects that reported a positive association between BCAA concentrations and markers of inflammation (Hamaya et al., 2021). Also, a recent study on pigs reported an increase in

some of the amino acids (phenylalanine, threonine, histidine, alanine, cysteine, and serine) with LPS challenge (Duanmu et al., 2022).

In addition, the similarity in amino acid concentration (essential, non-essential, and total) between groups in both experiments implies that the body compensates for the drop in some amino acids with the increase in others. This reshuffling in the amino acids to maintain a narrow concentration can be for the sake of preserving osmolarity. The current data infer that even under extreme lysine deficiency (60% lysine deficiency), which results in classical malnutrition manifestation, total amino acid concentration was maintained.

This work underscored an inverse association between albumin and CRP expression in the context of lysine deficiency. Lysine restriction at 60% was found to alter both plasma albumin and hepatic CRP expression. Lysine intake is thought to account for about 70% of the variations in plasma albumin concentrations. This can be explained by lysine requirements for albumin synthesis since lysine contributes to around 12% of albumin structure.

Lysine is the most dominant essential amino acid in the sequence of albumin (Denko et al., 1970), which is also prominent in CRP composition (Oliveira et al., 1979). Although lysine serves as a substrate for both albumin and CRP syntheses, an inverse relationship exists between these two proteins. Thus, it is not clear whether, under conditions of low lysine availability, the body prioritizes the production of small proteins requiring less lysine (CRP) at the expense of larger proteins (albumin, 3 times bigger than CRP). Herein, we found that 60% lysine deficiency alone decreased albumin levels by 20% and increased liver CRP expression by around 2.5 folds, independently from IL-6 and IL-1β, the main precursors of CRP (Sproston &

Ashworth, 2018; Zhang et al., 1996). Therefore, it is reasonable to postulate that the beneficial effect of dietary lysine is not related to its impact on growth only but rather to the improvement of albumin status and reduction of CRP, a sensitive marker of inflammation.

While several mechanisms can explain the inverse association between lysine intake and CRP expression, we hypothesize that lysine works as a regulator of CRP expression through the suppression of a transcriptional for the CRP gene in the liver. When lysine is sufficiently available, it stimulates the expression of a compound that binds to a transcription factor and forms a complex that inhibits the translational activity of the CRP gene. However, under lysine deficiency, the compound is poorly expressed, and the suppression of this transcriptional activity is lost, and so is the control over CRP transcription, thus expression (Figure 55). An example of this mechanism could be through the overexpression of Sirt1, which binds to hepatocyte nuclear factor-1 alpha (HNF-1α), a transcription factor for the CRP gene. The binding between Sirt1 and HNF-1α controls the transcriptional activity, a process suggested by Grimm et al. in response to overeating and fasting (Grimm et al., 2011).

The suppression seems to be dose-dependent as the drop in plasma lysine concentration with 30% lysine deficiency did not result in major alterations.

In parallel, lysine is the most abundant essential amino acid in the sequence of albumin and it is also of high importance for CRP. When lysine is deficient, and consequently CRP expression is stimulated, little substrate remains for the production of a large pool of albumin, thus albumin concentration drops (Figure 56).

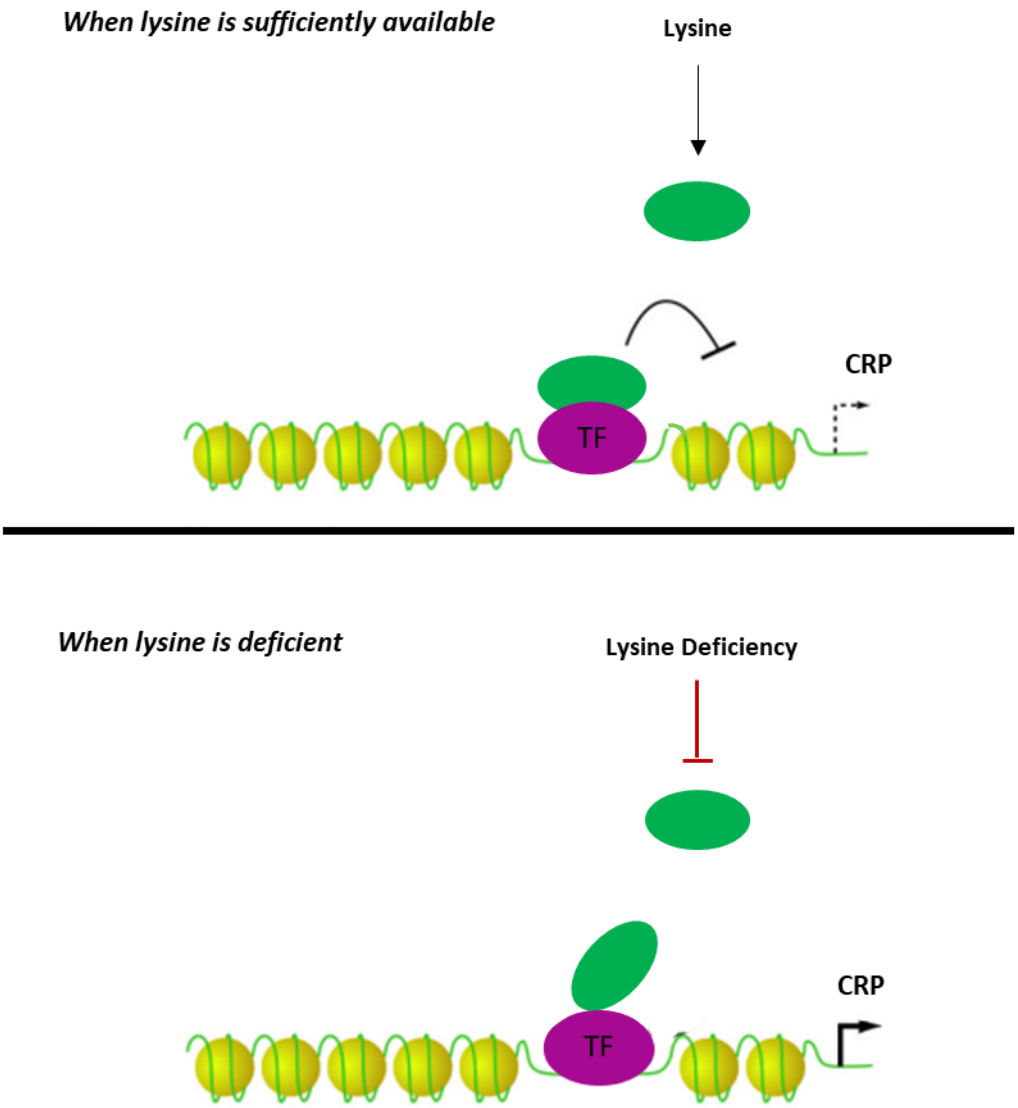

Figure 55. A postulated pathway for the interplay between lysine intake and availability and hepatic CRP expression
This figure is adapted from Grimm *et al.*, 2011.

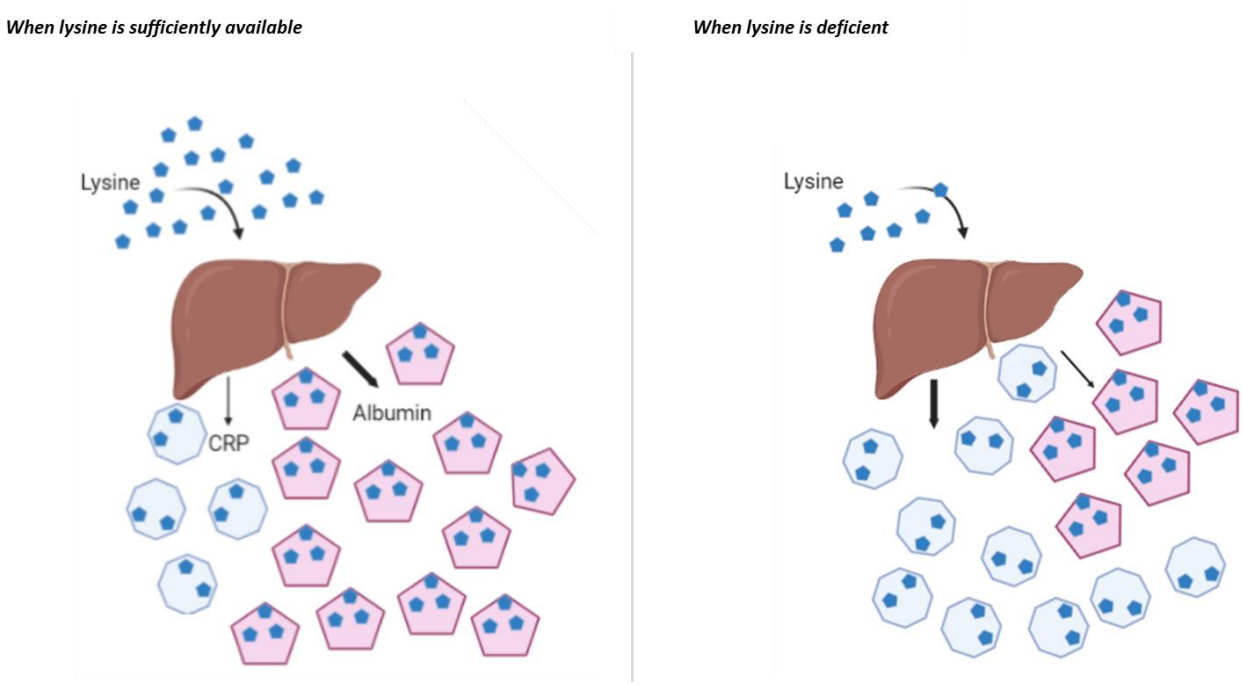

Figure 56. Low lysine availability decreases albumin production
Image created with BioRender.

Interestingly, the observed increase in albumin following LPS administration might correspond to the role of albumin as a carrier protein and immune modulator. In fact, albumin was shown to bind to LPS and form a much less effective complex in activating TLR4 than LPS-Lipid A (Arroyo et al., 2014). However, unexpectedly, hepatic CRP expressions did not increase with LPS despite the increase of some other inflammatory markers. Nonetheless, findings are not consistent in the literature. A study on pigs reported an increase in CRP levels 12 and 24 hours following an intravenous (IV) LPS injection (1.5 µg/kg) (Nordgreen et al., 2018). However, blood levels of CRP increased 1.8 times compared to the control group, 24 hours following an IP LPS injection of 1.5 mg/kg only in rats susceptible to hypoxia (Dzhalilova et al., 2019). Also, Mei et al. performed a study on broilers and injected the animals intraperitoneally

with as high as 500 µg/kg of LPS, a quantity close to the total amount given in 4 weeks in our study, 2 hours before tissue collection. They showed that hepatic mRNA expression of CRP did not change with LPS, yet serum amyloid A (SAA) increased 5 to 7 folds (Mei et al., 2020). This study suggested that SAA is a sensitive indicator of gram-negative bacterial infection. Similar results were also shown in the intestines of ulcerative colitis mouse model when cells were stimulated with LPS (Wakai et al., 2022).

An assessment of pain sensation in rats showed no difference among groups in both experiments, affirming that the results presented in the OFT were not caused by painful motion. The concentration of LPS used in our study was not enough to provoke pain, unlike what is commonly reported about LPS-inducing hyperalgesia (Davies & Hagen, 1997; Mason, 1993; Zouikr et al., 2014). Nonetheless, Yirmiya *et al.* demonstrated that the administration of LPS (200 µg/kg, which is 4 times the amount in this study in a single dose) increased pain sensitivity 2 hours after injection and lasted for around 30 hours (Yirmiya et al., 1994). Hence, the low dose of LPS (50 µg/kg) and/or the time of the test (24 hours after LPS injection) could have contributed to the negative results.

The OFT resulted in different outcomes implying that a small amount of lysine is sufficient for normal brain function since 30% lysine deficiency did not display any odd behavior. In contrast, 60% lysine deficiency increased the total distance and distance in the periphery traveled by the rats, suggesting hyperactive behavior. Additionally, rats on 60% deficient diets spent more time in the corners than rats on control diets, indicating an anxiety-like behavior. This result, seen in session 1 and

lost in the subsequent sessions, might be attributed to the habituation of the rats and their learning ability that was maintained.

The change in the rats' behaviors can be explained through several mechanisms. First, the 60%-lysine-deficiency-induced hyperactive behavior was associated with an increase in NEAT and energy expenditure. This could have been a regulatory mechanism to eliminate the accumulated amino acids. In support, protein malnutrition was reported to induce attention-deficit/hyperactivity disorder (ADHD) in rats (Salem et al., 2022). Second, lysine acts as a glutamate precursor in the mammalian central nervous system (Papes et al., 2001), and a selective reduction of glutamate in the ventral hippocampus is associated with anxiety-like characteristics in rats (Marrocco et al., 2012). Also, lysine can work as an antagonist of serotonin receptors in the brain and provide an anti-depressant effect, similar to the findings of Cremers *et al.* (Cremers et al., 2004). In a study on guinea pigs, lysine inhibited the binding of serotonin to its receptor 4 (5-HT4), reducing anxiety-related diarrhea (Smriga & Torii, 2003). Moreover, as a carrier protein, albumin binds to amino acids for their transport. Any decrease in plasma albumin level (by a reduction of production), availability (e.g., by an increase in free fatty acids), or both would increase the availability of free tryptophan (Yamamoto et al., 1997), altering the ratio of tryptophan to other large neutral amino acids (LNAA) that compete for transport across the blood-brain barrier (Zaragozá, 2020). Consequently, this imbalance changes the uptake of amino acids by the brain (Shulkin et al., 1995), which results in an alteration in the production of the varied neurotransmitters.

The link between behavioral disorders and inflammation has been extensively reported (Liu et al., 2021; Vogelzangs et al., 2013), arguing which came first, the

chicken or the egg. While both sides have valid arguments, we propose a reciprocal bidirectional relationship based on our results. The 60% lysine deficiency induced a hyperactive behavior, which was exacerbated with LPS administration. In other words, the presence of lysine in the control diet protected against the harmful effect of LPS on the brain. In support, in a chronic mild stress model in mice, low LPS dose induced aggression and hyperactivity and altered serotonergic function through serotonin transporter (SERT) upregulation (Couch et al., 2016). The mediator between peripheral inflammation and behavioral change is still controversial. While some authors believe that the increase in pro-inflammatory markers (such as IL-1β and TNF-α) in several brain structures is associated with alteration in the behaviors (Nordgreen et al., 2018; Sulakhiya et al., 2016), others consider that these cytokines exert minimal effects (Couch et al., 2016; Cunningham et al., 2009).

The strength of this project lies in its design and objectives. To our knowledge, this is the first study to determine the effect of varied levels of lysine deficiency on body and energy parameters, inflammatory profile, and behavior of male rats in the presence and absence of LPS challenge. However, we recognize several limitations in this work.

We acknowledge the importance of conducting the same study on female rats considering the discrepancies in serum albumin concentrations between sexes, especially at a young age (Weaving et al., 2016), and knowing that females have a compensatory mechanism that alleviates the response to endotoxins (Engler et al., 2016). These sex differences highlight a main limitation of our study.

Also, it is true that CRP is primarily produced by the liver, and protein expression in the liver would largely reflect blood levels. However, we do not deny other sources of CRP synthesis (macrophages, lymphocytes, other adipocytes than the epididymal) and would have preferred to have a clearer picture by assessing CRP or hsCRP in blood.

Another limitation would be the lack of SAA assessment that would support our findings and interpretation of results.

Lastly, a more robust conclusion about hyperactivity and anxiety-like behavior of the rats would need more than one behavioral test and perhaps a larger sample size. This part of the project must be built upon for future in-depth and elaborated work.

In conclusion, this work presents evidence of dose-dependent alterations associated with lysine deficiency, whereby a 30% reduction of dietary lysine resulted in milder signs of metabolic changes. In addition to the classical malnutrition signs, 60% dietary lysine deficiency hindered albumin production and stimulated an IL-6-independent hepatic CRP expression. These alterations were accompanied by an increase in hyperactivity and anxiety-like behavior and were exacerbated by LPS insult. This project adds to the recent advancements and understanding of the interplay between nutrition and inflammation, shedding light on amino acids and protein quality. The mechanistic pathway behind these alterations remains to be elucidated.

We believe that low SES populations who rely heavily on cereals as a main source of protein might be at risk of metabolic perturbations favoring a pro-inflammatory milieu and putting them at high risk of NCDs.

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