Concentration and ratio of essential fatty acids influences the inflammatory response in lipopolysaccharide challenged mice

K.J. Hintze[1,2], J. Tawzer[1], , and R.E. Ward[1,2]

[1]Department of Nutrition, Dietetics and Food Sciences, Utah State University, Logan, UT 84322

[2]Applied Nutrition Research, Utah Science Technology and Research Initiative (USTAR), Logan, UT 84322

Corresponding Author: Robert E. Ward, Nutrition, Dietetics and Food Sciences Department, UMC 8700, Utah State University, Logan, UT 84322 Tel: (435) 797-2153 Fax: (435) 797-2379 robert.ward@usu.edu

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Summary

The goal of this study was to evaluate the role of both the % of dietary, 18-carbon PUFA (2.5%, 5% and 10%) and the n-6:n-3 ratio (1:1, 10:1 and 20:1) on the acute inflammatory response. Mice were fed diets for 8 weeks and injected intraperitoneally with LPS to induce acute inflammation. After 24 hours mice were sacrificed and plasma cytokines measured. Diets significantly affected the erythrocyte PUFA composition and the effect of PUFA ratio was more prominent than of PUFA concentration. The % dietary PUFA affected feed efficiency ($p<0.05$) and there was a PUFA × ratio interaction with body fat ($p<0.01$). In mice fed high %kcal from PUFA, those given a low n-6:n-3 ratio had more body fat than those fed a high ratio. Of the twelve cytokines measured, eleven were significantly affected by the % PUFA ($p<0.05$), whereas five were affected by the ratio ($p<0.05$). For seven cytokines, there was a significant PUFA × ratio interaction according to a two way ANOVA ($p<0.05$). These data indicate that dietary polyunsaturated fatty acids can affect LPS induced-inflammation.

Keywords
Omega-3
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Inflammation
Lipopolysaccharide (LPS)

1. Introduction

Essential fatty acids are required nutrients in animal diets because they cannot be synthesized *de novo*. The two 18 carbon essential fatty acids (EFA), linoleic acid (18:2n-6; LA) and linolenic acid (18:3n-3; ALA) can be further elongated and desaturated to create long chain unsaturated fatty acids such as arachidonate (20:4n-6; AA), eicosapentaenoate (20:4n-3; EPA) and docosahexenoate (22:6n-3; DHA) [1]. As the typical American diet contains a substantial excess of LA (n-6) over ALA (n-3), there is a greater proportion of AA (n-6) compared to EPA (n-3) in tissue long chain PUFA, which may influence inflammatory processes [2]. The 20 carbon PUFA species AA, EPA and dihomo-gamma-linoleate (20:3 n-6; DGLA) serve as signaling molecules (eicosanoids) when they are enzymatically released from membranes [3]. In response to stimuli, the fatty acid is cleaved from the cell membrane via a phospholipase (such as phospholipase A2)[4]. The process by which these 20 carbon fatty acids are recognized and cleaved from the membrane seems to be nonspecific for the particular class (n-6 vs. n-3) and thus the fatty acids released reflect their relative membrane proportions. The 20 carbon PUFA can then be acted upon by cyclooxygenases (COX) or lipoxygenases (LOX) which result in prostaglandins, prostacyclins and thromboxanes (COX metabolites) or leukotrienes (LOX metabolites). The net effect of eicosanoid release is complex, as it depends on the relative proportions of the different eicosanoids present, as well as the sensitivity of the tissues that sense them. In general, the eicosanoids generated from arachidonic acid (AA) are considered to be more potent mediators of inflammation than those generated from EPAs [5]. However, even COX metabolites of AA, such as PGE_2 can have both pro-inflammatory and anti-inflammatory activity [5]. Thus, dietary ratios of the

EFAs directly impact tissue structural lipid composition and consequently systemic inflammatory and immune processes [2].

One reason intakes of EFA are so high in the US that replacing saturated fatty acids with EFA in the diet leads to lower total and LDL cholesterol levels, with LA being the most effective fatty acid in achieving this effect [6]. Thus, dietary advice given to Americans has been to replace saturated fats with foods containing more EFA. Over the last 100 years, an increase of vegetable oils in Western diets has affected both total PUFA intake and the n-6:n-3 ratio. Blasbalg and colleagues [7] estimated that LA consumption increased from 2.23 to 7.21% of daily calories from 1909 to 1999 and in this same time frame, the ratio of dietary n-6 to n-3 fatty acids changed from 5.4 to 9.6. It has been suggested that the increased LA intake and high n-6:n-3 ratio may negatively affect health through excessive eicosanoid signaling [8].

Adding n-3 PUFA to the diet is generally considered as an effective strategy for reducing inflammation, and these molecules may affect inflammation in several ways (reviewed by Calder [4]). For example, high dietary intake of EPA results in the displacement of AA in membrane phospholipids which reduces the pool of available AA for eicosanoid synthesis. In addition, EPA inhibits the hydrolysis of AA from immune cell membranes. Furthermore, when fatty acids are cleaved from membranes enriched in EPA by PLA_2, the freed EPA competes with free AA for conversion by COX and LOX enzymes. Lastly, EPA-derived eicosanoids compete with those derived from AA at target cell binding sites, and the individual eicosanoids have different effects. While the rationale behind replacing AA with EPA is based on the weaker activity of the eicosanoids derived from EPA, there are also metabolites from EPA as well as DHA that actively promote inflammation resolution.

Specialized pro-resolving mediators (SPM) is a term for lipid mediators derived from EPA and DHA that promote the active resolution of inflammation and SPM includes resolvins, protectins, and maresins [9]. Omega-3 fatty acids may also induce anti-inflammatory effects in macrophages and mature adipocytes via GPR120 signaling [10]. [11] In addition, long chain n-3 PUFA enhance B cell-mediated immunity in mice fed both control and high fat diets [11].

Most studies that have examined the inflammatory potential of dietary PUFA have focused on long chain PUFA (EPA and DHA) interventions that may not be relevant to modern, western populations. For example, EPA and DHA intake is estimated to account for less than 0.05% of daily caloric intake compared to ~8% of daily caloric intake from plant derived LA and ALA [7]. While there is a general consensus in the literature that high intakes of n-6 fatty acids can result in a pro-inflammatory state, there are few studies in which both n-6:n-3 ratios and total PUFA concentrations have been investigated together. Moreover, most studies investigating inflammatory potential of dietary PUFA have focused on long chain n-3 interventions.

The goals of this study were to determine if the total PUFA content of the diet and/or the n-6:3 ratio has an effect on the acute inflammatory response in C57BL6/J mice. Consequently, we formulated nine diets in which the fat source of the AIN-93G diet was modified to produce 3 different PUFA levels (2.5%, 5% and 10% of total kcal) at three ratios of n-6 to n-3 (1:1, 10:1 and 20:1) using fatty acids exclusively from plant sources. These PUFA concentrations, n-6 to n-3 ratios and dietary sources are physiologically relevant in the context of modern western diets, however long chain PUFA and animal fats were purposefully avoided as each may have complex actions on the inflammatory response. Mice

were then challenged with LPS, a stressor that is well-known to induce an acute inflammatory response.

2. Materials and Methods

2.1 Diet Formulation

Nine diets differing in total PUFA content and n-6 to n-3 ratios were formulated using the AIN-93G purified rodent diet. This diet has a caloric density of 3.8 kcal/g and derives 18.8 % kcal from protein, and 63.9 % kcal from carbohydrate and 17.2 % kcal from fat. Nine diets were formulated with three different PUFA levels (2.5%, 5% and 10%) at three different ratios (1:1, 10:1 and 20:1). To produce the different fat compositions, seven different vegetable oils were blended at various proportions (Table 1). Diets were produced by Harlan Laboratories (Madison, WI), and individual catalog numbers are provided in Table 1. To protect against lipid oxidation, all diets contained 14 mg/kg TBHQ and were stored at 4°C.

2.2 LPS Challenge

All animal experimental protocols were approved by the Utah State University Institutional Animal Care and Use Committee. Male 4-5 week old C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) (n=12) were fed the experimental diets ad-libitum for 8 weeks. Food intake and animal weights were determined weekly. Three days prior to sacrifice, body composition was determined by NMR (EcoMRI LLC, Houston, TX). After 8 weeks, mice were injected intraperitoneally with 5mg/kg lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, MO) 24-h prior to sacrifice and six mice were injected with saline. Mice were euthanized by

CO_2 asphyxiation, blood was collected via cardiac puncture, and plasma was isolated by centrifugation.

2.3 Plasma Cytokine Analysis:

Plasma cytokine analysis was performed using the Q-Plex™ Mouse Cytokine - Screen (16-plex) array (Quansys Biosciences, Logan, UT, USA). Cytokines analyzed include: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-17, MCP-1, IFNγ, TNFα, MIP-1α, GMCSF, and RANTES.

2.4 Fatty Acid Analysis

Lipids were quantified in diets and red blood cells using gas chromatographic analysis of fatty acid methyl ester derivatives (FAMEs). The direct derivitization method of O'Fallon [12] was used after adaption to smaller sample sizes. In short, diet or erythrocytes samples (~100mg) were mixed with 234 µl of 10N KOH in water and 1.77ml of methanol. Samples were vortexed for 30s and then incubated at 55 °C for 1.5h in a shaking water bath. Samples were removed every 20m and vortexed for 30s. Next, samples were cooled, and 193 µl of 24N H_2SO_4 was added. Samples were vortexed for 30s and then incubated at 55 °C for 1.5h in a shaking water bath with vortexing every 20m for 30s. Samples were cooled and 300 µl of hexane with 0.05% BHT was added. Samples were vortexed for 30s and then centrifuged at 1000 × g for 5min. The upper hexane layer was removed to a GC vial and analyzed according to the method of Zhou et al [13] using a Shimadzu GC2010 gas chromatograph with flame ionization detection (Shimadzu Corporation, Columbia, MD). Fatty acids were separated using an HP-88 column (Agilent Corporation, Santa Clara, CA). The retention time

for fatty acids and response factors were determined using GLC-463, a pure standard of fatty acid methyl esters (Nu-Chek Prep, Elysian, MN).

2.5 Statistical Analysis

All data were analyzed using a 2 way ANOVA with n6:n3 ratio and % dietary PUFA as the main effects. Cytokine data was normalized to plasma protein concentration, and $\log_{10}$ transformed to adjust for non-normal distribution. After log transformation the data was normally distributed allowing for parametric testing.

3. Results

3.1 Fatty Acid Composition of Diets

The fat sources used to create the experimental diets are shown in Table 1. The low PUFA diets contained significant proportions of low PUFA triglyceride sources, such as cocoa butter (CB) and olive oil (OO). On the other hand, the n-6 and n-3 content of the diets were primarily manipulated by including different proportions of corn oil (CO), cottonseed oil (CSO), flaxseed oil (FSO), sunflower oil (SFO), and soybean oil (SO).

The measured fatty acid composition of the experimental diets is shown in Table 2. The primary focus of the diet design was to produce diets that contained different concentrations of PUFA at different ratios, yet at the same time to keep the saturated fatty acid (SFA) content similar. Therefore, the major difference between the low PUFA diets (2.5% kcal) and the high PUFA diets (10% kcal) was in the monounsaturated fatty acid content (MUFA). Despite careful formulation of the diets, the target PUFA contents and n-6:n-3 ratios were not all achieved. Nonetheless, the diets do segregate into low, medium and high PUFA contents with low, medium and high n-6:n-3 ratios.

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185 *3.2 Food Consumption, Weight Gain and Body Composition*

186 The experimental diets did not significantly affect total food intake or weight gain over
187 the course of the study (Table 3). MRI data of body composition indicated there were no
188 differences in lean mass, water content, but there were differences in both the absolute
189 amount of fat (data not shown) and the relative amount (% body fat). The differences in
190 body composition were the result of fat mass, as there were no differences in the absolute
191 amount of lean tissue (data not shown). In addition, there was an effect of PUFA intake on
192 feed efficiency, which is the ratio of weight gained to food intake (Table 3; Figure 1A).
193 When expressed as % body fat, there was an effect of the %PUFA, the ratio and a PUFA ×
194 ratio interaction for the percent body fat of the mice after consuming the diets for 8 weeks
195 (Table 3; Figure 1B). Mice fed the 10% PUFA diet at the 20:1 n-6:n-3 ratio had significantly
196 less body fat compared to those fed the same amount of PUFA but at the 1:1 n-6:n-3 ratio (P
197 < 0.05).

198

199 *3.3 Effects of Diets on Red Blood Cell Fatty Acid Composition*

200 The effects of PUFA concentration and the n-6:n-3 ratio on RBC fatty acid content are
201 shown in Table 4. Of the 19 fatty acids measured, 10 were significantly affected by the total
202 percent PUFA in the diets, whereas 12 were affected by the n-6:n-3 ratio. According to the
203 two-way ANOVA analysis, there was a PUFA × ratio interaction for four long chain
204 polyunsaturates (20:4n-3, 22:5n-6, 22:5n-3 and 22:6n-3).

205 The five most prominent fatty acids in the diets (16:0, 18:0, 18:1n9, 18:2n6 and 18:3n3;
206 Table 2) made up between 97-98% of the dietary fat and were variable across the diets.

Thus, it is not surprising all five were significantly different in the RBCs due to the PUFA percentage of the diets. One fatty acid of interest that decreased as a percentage of total RBC fatty acids with increased dietary PUFA was 20:3n9, Mead's acid. This fatty acid is produced via elongation of oleic acid at low PUFA intakes and has been suggested as a potential biomarker for low PUFA intakes [8].

As virtually all the fatty acids in the diets were <18 carbons, those in the RBCs with >18 carbons were primarily synthesized via elongation and desaturation of dietary precursors. Of the thirteen fatty acids with >18 carbons, five were significantly different according to the %PUFA whereas eleven of the thirteen were different according to the n-6:n-3 ratio (Table 4).

A post-hoc analysis was conducted on those fatty acids for which there was a %PUFA × ratio interaction and the results are shown in Figure 2. For EPA (20:5n-3) there were significant differences between the mice fed the 1:1 diets and those fed the 10:1 and 20:1 diets. In addition, the EPA percentage of the RBCs increased at the 1:1 ratio when the mice were diets contained 5% PUFA (2.5% n-3) compared to the 2.5% PUFA(1.25% n-3), but did not increase further when the PUFA content was raised to 10% (5% n-3). The RBC percentage of Osbond acid (22:5n-6) increased with the n-6:n-3 ratio. Similar to EPA, there was an increase in the concentration when the PUFA content of the diet was increased from 2.5% to 5%, but no further increase when the PUFA was at 10%. The RBC percentage of clupanodonic acid (22:5n-3) was higher in the low ratio diets (1:1) but increased as the dietary PUFA went from 2.5% of kcal to 10%. Lastly, the RBC DHA content (22:6n-3) did not differ at the low ratio (1:1) regardless of the %kcal from PUFA, but was lower at higher n-6:n-3 ratios.

The omega-3 index is the sum of EPA and DHA in red blood cells, and the average values for mice in each diet group are shown at the bottom of Table 4. s According to the two way ANOVA, there is both a %PUFA and a ratio effect, but no interaction. Mice fed the 1:1 ratio of n-6:n-3 had omega-3 indices over 10, while those fed the 10:1 ratio were between 4.7 and 6.2. Mice fed the high n-6:n-3 ratio diets had the lowest omega-3 indices between 3.3 and 4.6.

3.4 Effect of Diets on LPS Induced Systemic Inflammation

Plasma cytokine data from mice given the sham injections is not shown, but there were few statistical differences between groups. Conversely, levels of all cytokines were significantly higher in mice treated with the 5 mg/kg LPS. This dose was selected to induce a vigorous, yet non-lethal inflammatory response. In a previous study in our lab, 10 mg/kg was lethal to mice in some diet groups but not others [14], despite the fact that it is regularly used to induce non-lethal inflammation [15]. Due to the distribution of the raw data, all cytokine data was log transformed prior to statistical analysis. For presentation in the figures, the data was back transformed, and is shown in mass per mg protein. Of note, IL-6, RANTES and MCP-1 are all in ng/mg protein, whereas the rest are in pg/mg protein.

Cytokines significantly affected by the dietary PUFA modulations are shown in Table 5 and Figure 2. According to the data, 11 of the 16 cytokines measured were significantly affected by the %PUFA content of the diet, whereas 5 of 16 responded to changes in the n-6:n-3 ratio. Furthermore, with 7 cytokines there was a ratio × concentration effect. The first five cytokines shown in Figure 2 (INFγ, IL-5, IL-6, IL-10 and IL-12p70) were affected by the %PUFA content of the diet, but not the ratio. For these cytokines, comparison is made at each

253 ratio, and significant differences are designated by lower case letter, and boxes are placed
254 around groups within each ratio. For example, for INFγ, increasing the %PUFA of the diets
255 led to significant differences at each ratio. Thus, there was a greater INFγ response at ~10%
256 PUFA than at 2.5% regardless of ratio. For IL-5, the response was greater in mice fed
257 10%PUFA at the 1:1 ratio compared to mice fed 2.8% PUFA. At the 10:1 ratio mice fed the
258 10% PUFA diets had a greater IL-5 response than mice fed the 2.5 and 5% PUFA diets. Lastly,
259 for the high ratio, increasing the %PUFA content of the diet led to a more robust IL-5
260 response at each level.

261 The IL-6 response to LPS was primarily driven by the %PUFA as well. At each ratio,
262 increasing the PUFA of the diet resulted in a more robust IL-6 response. However, there was
263 no ratio effect and thus, the IL-6 response in the high PUFA diets was not different between
264 the mice fed the 1:1 and 10:1 ratios at any PUFA level. For IL-10, the only significant
265 differences were at the high ratio. Mice fed the 2.5% PUFA diet had a less robust IL-10
266 response compared to mice fed the 5% and 10% PUFA diets. For IL-12p70 the results are
267 similar to IL-5 and IL-6. Increasing the %PUFA content of the diet resulted in more IL-12p70,
268 but only at the ~10% PUFA intakes.

269 For the remaining seven cytokines measured, there was a %PUFA × ratio interaction and
270 the post-hoc statistical analysis compared all diet groups. Consequently, in Figure 2, the
271 arrangement of diet groups for the last seven cytokines is by %PUFA along the bottom axis.
272 For IL-1β, there was a diminished response at the low PUFA level. Interestingly, mice fed the
273 1:1 ratio at 10% PUFA had a greater response than mice fed the 2.5% or 5% PUFA. For IL-
274 17, the only differences were between the 2.5% PUFA and 16:1 ratio and the 10:1 and 24:1
275 ratios at 10% PUFA. The IL-3 response pattern among diets was unique. Mice fed the 1:1

ratio at 10% PUFA had a more robust response than all other diet groups. Conversely, for MIP1α, mice fed the 1:1 ratio at 10% PUFA had a lower response compared to mice fed the 10% PUFA at the 10:1 and 24:1 ratio. The response of TNFα was qualitatively similar to MIP1α, in mice fed the 1:1 ratio at 10% PUFA had a lower response than mice fed the 10:1 and 24:1 ratios. The other group that had a low expression of TNFα was for mice fed the low 2.5% PUFA diet at a 16:1 ratio.

The RANTES response to the LPS challenge was similar across all diet groups, except mice fed the low ratio, high PUFA diet. Lastly, for MCP-1, there were no differences at the low and medium PUFA intakes, but dramatic effects at the high PUFA level. At ~10% PUFA, a 10:1 ratio resulted in the greatest response, followed by the 24:1 and 1:1 ratios.

4. Discussion and Conclusions

The goal of this study was to determine how the %kcal from LA and ALA PUFA and the n-6:n-3 ratio affect the acute inflammatory response to intraperitoneal LPS injection in mice when provided on the background of the AIN-93G diet. The fat composition of the diets had a significant impact on feed efficiency and body composition despite the short feeding time. The fat composition of the diets significantly affected the RBC composition, with the 1:1 ratio diets resulting in the largest values for the omega-3 index. LPS administration caused a significant increase in all 12 cytokines measured, compared to the sham injected mice, and there were significant differences between mice fed different fat formulas. Some cytokines responded only to the %PUFA level, some to the ratio, and for some there was a %PUFA × ratio interaction.

The higher level of body fat in mice fed the 10% PUFA diet at the 1:1 ratio, compared to the 24:1 ratio was a surprise. This effect was present on both an absolute and percent body composition basis. In addition, according to the MRI data, there were no differences between groups in either lean mass, or water content, which indicates the diets did not restrict growth. One reason this result was unexpected is that it has previously been shown in C57BL/6J mice that high LA diets induce adipocyte differentiation via prostacyclin signaling of AA metabolites [16]. Yet, according to Table 4, the mice with more adipose (10%PUFA, 1:1 ratio) had substantially less AA in their RBC membranes than mice fed the 10% PUFA diets with a 24:1 n6:n3 ratio. One significant difference between their design, and ours, is that in the present study the diets only provided 17% of energy as fat, whereas in the study cited above it was 40%.

Hanbauer et al. showed that a high n-6:n-3 ratio fed over three generations will induce obesity [17]. The low n-6:n-3 ratio diet contained 9.68% LA and 1.02% ALA, whereas the high ratio diet was 12.31% LA and 0.16% ALA. Thus, the n-6:n3 ratios were 9.5:1 and 77:1. Interestingly, the diets were less energy dense than ours, as their diets were 3.1 and 3.2 kcal/g wherease ours were 3.9 kcal/g. One other notable difference is that they used B6C3Fe mice. Massiera et al. also showed that mice fed a high n-6:n-3 ratio diet became gradually more obese over four generations [18]. However, one major issue with this study is that the high n-6 diet had 3X the amount of fat as the control diet. Thus, it is not clear what the primary driver of fat mass was over successive generations.

Alvheim et al. also found that increasing the dietary intake of LA was associated with more adipose gain [19]. C57BL/6j mice were fed either moderate (35% kcal) or high-fat (60% kcal) diets for 14 weeks. The diets contained either 1% or 8% of calories as LA, and ALA was

held constant at 1% of calories. In addition, a third group was fed 8% LA and had an additional 1% of a mixture of EPA and DHA. Their results indicated that high LA induced obesity via increases in the proportion of AA in liver and erythrocyte phospholipids and associated endocannabinoid signaling. In a separate study using similar LA and ALA treatments on a low fat diet, Alvheim et al found that 8% LA caused greater weight gain over 1% ALA. However, there were no differences when the same PUFA treatments were presented in medium fat diets (35% kcal from fat). In both the studies by Alvheim, higher intakes of LA increased feed efficiency. We found a %PUFA effect on feed efficiency, but no ratio effect (Table 3; Figure 2). Other groups have not found an effect of high LA intakes and high n-6:n-3 ratios on obesity and weight gain. For example, Enos et al. fed mice diets composed of 40% kcal by fat where n-6 fatty acids comprised 4.7%, 7.8%, 8.6% and 9% kcal [20]. By also changing the n-3 content the diets had 1:1, 5:1, 10:1 and 20:1 ratios of n-6 to n-3. In contrast to the other studies described above, there was no effect of the different fat formulas on weight gain, visceral adipose mass, or adipocyte size, despite significant effects on the ratios of AA and both EPA and DHA in adipose phospholipids. While there may be an effect of higher LA intakes on weight gain and adiposity in some animals and on some diet backgrounds, the results are not consistent.

Populations that consume increased amounts of n-3 fatty acids appear to receive protection from some pathologies associated with inflammation, advice given to individuals is to change the n-6 to n-3 ratio by eating more oily fish, and other foods rich in n-3s like flax seed oil. In the diet of early humans, the estimated ratio of n-6 to n-3 fatty acids was 0.8:1, yet in some contemporary populations the ratio has increased to ~17:1 [21], presumably due to the increased consumption n-6 rich vegetable oils and decreased fish consumption

[2]. In our LPS challenge model, increased dietary PUFA concentration had a strong stimulatory effect on several plasma cytokines regardless of n-6 to n-3 ratio including IL-1β, IL-5, IL-6, IL-10, IL-12p70, IL-17 and INF-γ and the dietary n6n-6 to n-3 PUFA ratios affected MCP-1, TNF-α, MIP-1α and RANTES. Our data suggests that the total amount of dietary PUFA may be an important determinant in the acute LPS inflammatory response and the n-6 to n-3 ratio may only be important at higher concentrations of total dietary PUFA.

In recent years it has been suggested that a high n-6 intake and high n-6:n-3 ratio promote the pathogenesis of many diseases [20]. The link between increased n-6 intake, inflammation and disease progression is based on the idea that higher dietary intakes of LA will increase cellular pools of AA, and will promote a more inflammatory cytokine response [21]. Yet, despite this supposition, in humans consuming Western-type diets, plasma AA is resistant to large changes in dietary LA [22]. In addition, while human peripheral blood mononuclear cell PUFA composition does affect immune function, the LA, ALA, AA, EPA and DHA levels do not necessarily correlate with dietary intake [23]. Furthermore, in a cross-sectional analysis of plasma PUFA levels and inflammatory markers, the lowest quartile of n-6 PUFA was associated with the highest concentration of TNFα and Il-6 [24]. Because the n-6:n-3 ratio is driven by both n-6 and n-3 consumption, effects will be affected by both the total and relative intakes.

Several studies have investigated the effects of dietary n-3 on systemic inflammation but the focus of the majority of these studies has been on long chain n-3 fatty acids, primarily EPA and DHA. Our data suggest that dietary intake and relative ratios of plant derived n-6 and n-3 fatty acids have profound effects on the LPS induced inflammatory response. Very few studies have investigated the effects of dietary n-6 and n-3 fatty acids on acute, LPS

initiated inflammation and to our knowledge, no studies have investigated the relative contributions of both total PUFA concentration and ratios of n-6 to n-3. Chavali et al. [22] injected Balb/c mice with a lethal dose (20 mg/kg) of LPS. Mice were fed either safflower oil diets or safflower + linseed oil diets. The fat content of the safflower oil diets was 79.4% LA or ~24% of total energy with negligible ALA. The safflower + linseed oil diet contained approximately equal LA and ALA, approximately 12% of total dietary energy for each fatty acid. Plasma levels of IL-6 were higher in the LA fed animals compared to the LA + ALA fed mice while TNF-α levels were higher in the LA + ALA animals. Although in our study much lower levels of dietary PUFA were fed, we found that that decreasing the n-6 to n-3 ratio by incorporating dietary ALA decreases LPS induction of plasma TNF-α. In a previous LPS challenge study by our group [23], mice were fed either the AIN-76 diet or a modified AIN-76 diet that replaced the corn oil fat source with dairy lipids. The dairy lipid diet contained only 0.6% of calories from PUFA and had had an n-6 to n-3 ratio of 9.4. Conversely, the corn oil fat sourced AIN-76 diet contained 6.9% of calories from PUFA and n-6 to n-3 ratio of 56.4. Similar to the current study, mice fed the lower PUFA, lower n-6 to n-3 ratio diet had significantly lowered levels of plasma IL-6, IL-3, IL-10, IL-17, MCP-1, IFN-γ, TNF-α and IL-12p70.

The effects of dietary LA and ALA have been investigated in other models of acute inflammation, including chemically induced colitis. In a recent study [24], colitis was induced in rats fed either corn oil or an ALA rich camelina oil diet. Rats fed the ALA rich diet had lowered colonic expression of IL-1β and TNF-α. Similarly, in a study by Tyagi and coworkers [25] rats were fed differing n-6 to n-3 ratios by manipulating dietary LA and ALA levels varying from 215:1, 50:1, 10:1 and 2:1. Inflammatory colitis was induced by dextran

sodium sulfate treatment. Colonic expression of both IL-1β and TNF-α was lowest at the 2:1 ratio. In our study, the n-6 to n-3 ratio significantly affected plasma levels of TNF-α however, plasma IL-1β were not responsive to the PUFA ratio and increased as a function of total dietary PUFA.

The inflammatory response to LPS is mediated through the LPS receptor, Toll-like receptor 4 (TLR4). Circulating LPS is bound by the secreted LPS binding protein (LBP) and then can form a complex with CD14 and interact TLR4 to initiate a signaling cascade that results in the degradation of IκB and binding of NF-κB to the promoters of immune response genes [26]. NF-κB is a ubiquitously expressed transcription factor that mediates the inflammatory response to a diverse set of signals including LPS. Activation of NF-κb involves translocation from the cytosol to the nucleus after disassociation from the inhibitory subunit IκB. The resulting signaling causes increased expression of several inflammatory cytokines including TNF-α, IL-1β, IL-6 and INF-γ. Fatty acids are known to play a role in the NF-κB signaling pathway. Zhao et al (2004) demonstrated that EPA inhibited NF-κB inflammatory activation by preventing the phosphorylation of IκB in THP-1 cells and inhibited NF-κb activation similar to DHA in LPS induced THP-1 cells [27, 28]. Inhibition of NF-κB activation by fish oil has also been reported in RAW 267.4 macrophages [28]. Lee and coworkers [29] demonstrated that DHA and EPA are the most potent inhibitors of LPS induced NF-κb activation in RAW 264.7 mouse monocytes. However, arachidonic, linoleic and oleic acid also inhibited NF-κb activation compared to cells treated with lauric acid. Fatty acids are also thought to influence inflammatory pathways by acting as ligands for PPARγ. PPARγ is expressed in many tissues including adipose, muscle, and vascular cells. Activated PPARγ induces lipoprotein lipase and fatty acid transporters and enhances adipocyte differentiation

as well as inhibiting NF-κB function, cytokine and COX-2 expression. Acting as a PPARγ ligand, EPA has been demonstrated to attenuate expression of IL-6 in C6 glioma cells [30] and Caco-2 intestinal cells [31].

It is not clear why the diets lowest in PUFA generally resulted in less inflammation in the current study. Animals fed the 2.5% PUFA diets at the 10:1 and 24:1 rations did not have lower levels of AA or higher levels of EPA or DHA as RBC fatty acids compared to the 10% PUFA diets. Therefore, our data suggests that the reduced inflammation observed in the low PUFA compared to the high PUFA diets may be independent from conversion of LA to AA or ALA to EPA and DHA.

Taken together, our data suggest that the most commonly consumed n-3 and n-6 fatty acids in modern diets, LA and ALA, play an important role in the susceptibility to acute inflammatory stressors. Current public health recommendations have stressed the importance of lowering the n-6 to n-3 ratio and incorporating long chain n-3 fatty acids such as EPA and DHA into diets. By designing a study to determine the inflammatory potential of both dietary PUFA concentration and n-6 to n-3 ratio using PUFA sources relevant to modern diets; we have found that total dietary PUFA concentration must be considered as an important factor in acute inflammation. This finding may have implications for conditions such as sepsis and bacterial infections. However, the molecular mechanisms as to how different concentrations and ratios of dietary LA and ALA influence LPS mediated inflammation are not known. Future work examining the effects of these diets on candidate pathways such as expression of toll like receptors, NF-κB and PPAR-γ, and on PUFA-derived lipid mediators is warranted.

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REW and KJH were involved in the study design. REW designed the diets and KJH and JT conducted experiment 1. BBG and JH conducted experiment 2. REW, KJH and ML performed the data analysis and interpretation. KJH and REW wrote the manuscript and all authors contributed to producing the final manuscript.

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586

Table 1: Fat formulas for experimental diets. Values are expressed as a percentage of total dietary fat. Abbreviations are: Cocoa butter (CB), corn oil (CO), cottonseed oil (CSO), flaxseed oil (FSO), olive oil (OO), sunflower oil (SFO) and soybean oil (SO). The diets were prepared by Harlan Laboratories (Madison, WI) and the catalog numbers are given in the table.

Diet	Target %PUFA	n-6:n-3 Ratio	CB	CO	CSO	FSO	OO	SFO	SO	Catalog #
1	2.5	1:1	45.5	--	--	12.5	42.0	--	--	TD.10148
2	2.5	10:1	29.5	11.0	--	15.5	58.0	--	--	TD.10149
3	2.5	20:1	23.0	9.5	--	--	65.0	--	2.5	TD.10150
4	5	1:1	45.0	--	--	23.0	15.5	--	16.5	TD.10151
5	5	10:1	26.2	39.0	--	3.8	31.0	--	--	TD.10152
6	5	20:1	15	--	30.0	--	42.0	--	13.0	TD.10153
7	10	1:1	8.0	--	40.0	52.0	--	--	--	TD.10154
8	10	10:1	--	--	79.4	9.1	--	11.5	--	TD.10155
9	10	20:1	--	--	65.5	4.5	5.0	25.0	--	TD.10156

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Table 2. Fatty acid composition of diets. Values are expressed as a percentage of total dietary energy.

	Target Values		*Measured Values*								
Diet	**PUFA%**	**Ratio**	**16:0**	**18:0**	**18:1n9**	**18:2n6**	**18:3n3**	**SFA**	**MUFA**	**PUFA**	**ratio**
1	*2.5*	*1:1*	2.8±.1	2.8±.1	8.4±.1	1.5±.1	1.4±.1	5.6	8.4	*2.8*	*1:1*
2	*2.5*	*10:1*	2.5±.1	2.0±.2	9.8±.1	2.3±.1	0.3±.1	4.5	9.8	*2.5*	*9:1*
3	*2.5*	*20:1*	2.4±.1	1.7±.1	10.2±.1	2.4±.1	0.2±.1	4.1	10.2	*2.5*	*16:1*
4	*5*	*1:1*	2.6±.1	3.5±.2	6.2±.4	2.6±.4	2.0±.7	6.1	6.2	*4.5*	*1:1*
5	*5*	*10:1*	2.4±.1	2.0±.3	7.5±.2	4.5±.3	0.5±.1	4.4	7.5	*5*	*10:1*
6	*5*	*20:1*	2.6±.1	1.4±.2	7.9±.2	4.8±.3	0.3±.1	3.9	7.8	*5*	*18:1*
7	*10*	*1:1*	2.2±.1	1.1±.3	3.7±.4	5.3±.2	4.8±.7	3.3	3.7	*10*	*1:1*
8	*10*	*10:1*	2.8±.1	0.5±.1	3.4±.1	9.2±.2	0.9±.1	3.3	3.4	*10*	*10:1*
9	*10*	*20:1*	2.7±.2	0.8±.2	4.5±.5	8.5±.8	0.4±1	3.5	4.5	*8.8*	*24:1*

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Table 3: Results of two way ANOVA for food intake, weight gain, and body composition.

	% PUFA	Ratio	Interaction
	p value		
Food intake	0.46	0.11	0.34
Weight gain	0.08	0.36	0.12
Feed efficiency	**0.04**	0.43	0.13
Percent body fat	0.06	**<0.01**	**<0.01**

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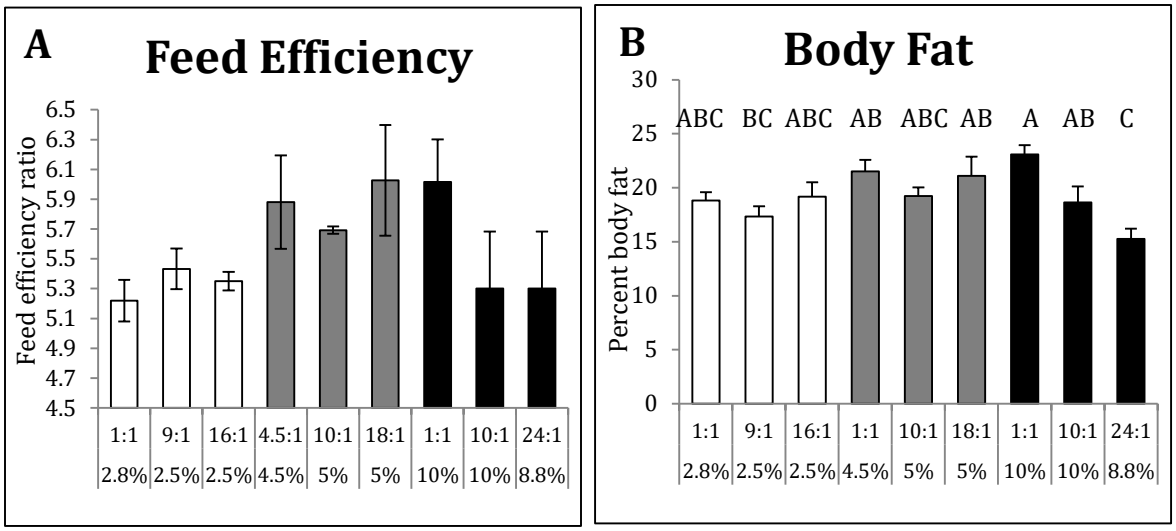

Figure 1: Feed efficiency ratio (A) and percent body fat (B) of mice fed nine experimental diets. There was a significant effect of PUFA on the feed efficiency ratio (p=0.4), but no n-6:n-3 ratio effect or interaction in the two way ANOVA (A). For body fat there was both a ratio effect (p<0.01) and a PUFA × ratio interaction (p<0.01) as determined by the two way ANOVA. Groups with the same letter are not significantly different according to the Tukey HSD test.

Table 4: Fatty acid composition of erythrocytes (in percent) from mice fed 9 diets differing in %PUFA and the n-6:n-3 ratio.

PUFA %kcal	2.8%	2.5%	2.5%	4.5%	5%	5%	10%	10%	8.8%	*p* values for 2 × 2 ANOVA		
n-6:n-3 ratio	1:1	9:1	16:1	1:1	10:1	18:1	1:1	10:1	24:1	PUFA	Ratio	Interaction‡
16:0	25.2±0.6	24.6±1.2	24.1±0.2	26.3±0.5	24.4±0.8	24.8±0.5	26.1±0.7	27.3±2.1	26.5±1.4	***<0.01***	NS	NS
16:1n-7	2.6±0.7	2.0±1.0	2.5±0.4	1.7±0.3	2.3±0.3	2.2±0.1	2.3±0.6	2.4±0.5	2.0±0.8	NS	NS	NS
18:0	12.6±0.3	12.6±0.3	12.3±0.3	13.7±0.1	13.6±0.6	13.4±0.2	13.6±0.7	12.6±0.8	13.0±0.1	***<0.01***	NS	NS
18:1n-9	22.3±1.4	21.6±1.4	21.3±0.7	17.4±0.1	17.3±0.5	18.0±0.4	14.3±0.6	12.3±0.8	11.9±0.4	***<0.01***	***0.04***	NS
18:2n-6	6.1±0.3	6.2±0.4	6.5±0.3	8.8±0.2	7.8±0.6	8.0±0.1	11.7±0.4	11.6±0.6	12.0±0.4	***<0.01***	NS	NS
18:3n-3	1.4±0.2	1.1±0.6	1.0±0.1	1.6±0.2	1.6±0.2	1.4±0.3	2.0±0.5	1.8±0.7	1.8±0.6	***<0.01***	NS	NS
20:0	0.4±0.1	0.5±0.2	0.7±0.4	0.2±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.2	NS	NS	NS
20:1n-9	0.6±0.2	0.8±0.2	0.7±0.1	0.4±0.2	0.8±0.3	0.5±0.1	0.4±0.1	0.8±0.3	0.7±0.4	NS	***0.03***	NS
20:3n-9	0.8±0.1	1.2±0.1	1.1±0.1	0.2±0.1	0.3±0.01	0.3±0.1	0.2±0.1	0.3±0.1	0.2±0.2	***<0.01***	***<0.01***	NS
20:3n-6	1.4±0.2	1.7±0.1	1.8±0.3	1.3±0.2	1.6±0.1	1.5±0.1	1.4±0.1	1.7±0.1	1.6±0.3	NS	***<0.01***	NS
20:4n-6	12.1±0.8	18.3±1.0	19.6±0.5	12.5±0.3	18.9±0.6	19.8±0.4	11.8±0.4	17.7±0.5	18.8±0.8	***0.02***	***<0.01***	NS
20:5n-3	2.3±0.3^{B}	0.3±0.1^{C}	0.2±0.1^{C}	2.9±0.1^{A}	0.5±0.5^{C}	0.1±0.02^{C}	3.0±0.2^{A}	0.3±0.1^{C}	0.1±0.02^{C}	***0.03***	***<0.01***	***0.01***
22:0	0.2±0.1	0.2±0.1	0.1±0.1	0.1±0.01	0.4±0.4	0.1±0.01	0.05±0.06	0.3±0.2	0.1±0.1	NS	***0.04***	NS
22:4n-6	0.7±0.1	2.1±0.2	2.2±0.1	0.6±0.4	2.2±0.1	2.4±0.1	0.6±0.1	2.0±0.2	2.7±0.4	NS	***<0.01***	NS
22:5n-6	0.2±0.02^{D}	1.7±0.1^{C}	2.2±0.2^{B}	0.2±0.03^{D}	1.5±0.1^{C}	2.7±0.2^{A}	0.1±0.04^{D}	1.5±0.1^{C}	2.7±0.2^{A}	NS	***<0.01***	***<0.01***
22:5n-3	1.7±0.1^{C}	0.4±0.1^{D,E}	0.2±0.1^{E}	2.2±0.1^{B}	0.5±0.4^{D,E}	0.3±0.03^{D,E}	2.7±0.1^{A}	0.7±0.02^{D}	0.5±0.1^{D,E}	***<0.01***	***<0.01***	***<0.01***
22:6n-3	8.9±0.8^{A}	4.4±0.4^{C}	3.2±0.1^{D}	9.6±0.1^{A}	5.4±0.5^{B,C}	3.8±0.2^{C,D}	9.1±0.3^{A}	6.0±0.3^{B}	4.5±0.4^{C}	***<0.01***	***<0.01***	***0.02***
24:0	0.2±0.03	0.2±0.1	0.1±0.03	0.2±0.1	0.2±0.0 3	0.2±0.03	0.2±0.01	0.2±0.03	0.2±0.04	NS	***0.01***	NS
24:1n-9	0.3±0.1	0.2±0.1	0.2±0.2	0.2±0.1	0.2±0.1	0.1±0.02	0.1±0.02	0.2±0.1	0.1±0.03	NS	NS	NS
Ω-3 index	11.3±1.0	4.7±0.4	3.3±0.1	12.5±0.1	5.9±0.9	3.8±0.2	12.0±0.4	6.2±0.4	4.6±0.4	***<0.01***	***<0.01***	NS

‡The 2 × 2 ANOVA indicated there were four fatty acids that had a %PUFA × ratio interaction (20:5n3, 22:5n6, 22:5n3, and 22:6n3). Superscripts indicated fatty acids that are significantly different according to the Tukey HSD test.

Table 5: Two-way ANOVA analysis of effects of dietary PUFA concentration and n-6:n-3 ratios on plasma cytokines in mice treated with LPS.

Cytokine	% PUFA	n-6:n-3 ratio	Interaction
INFγ	<0.01	NS	NS
Il-5	<0.01	NS	NS
Il-6	<0.01	NS	NS
Il-10	<0.01	NS	NS
ll-12p70	<0.01	NS	NS
IL-1β	<0.01	NS	0.02
Il-17	<0.01	NS	<0.01
Il-3	<0.01	<0.01	<0.01
MIP1-α	0.03	0.05	<0.01
TNFα	0.05	<0.01	<0.01
RANTES	<0.01	<0.01	<0.01
MCP-1	NS	<0.01	<0.01

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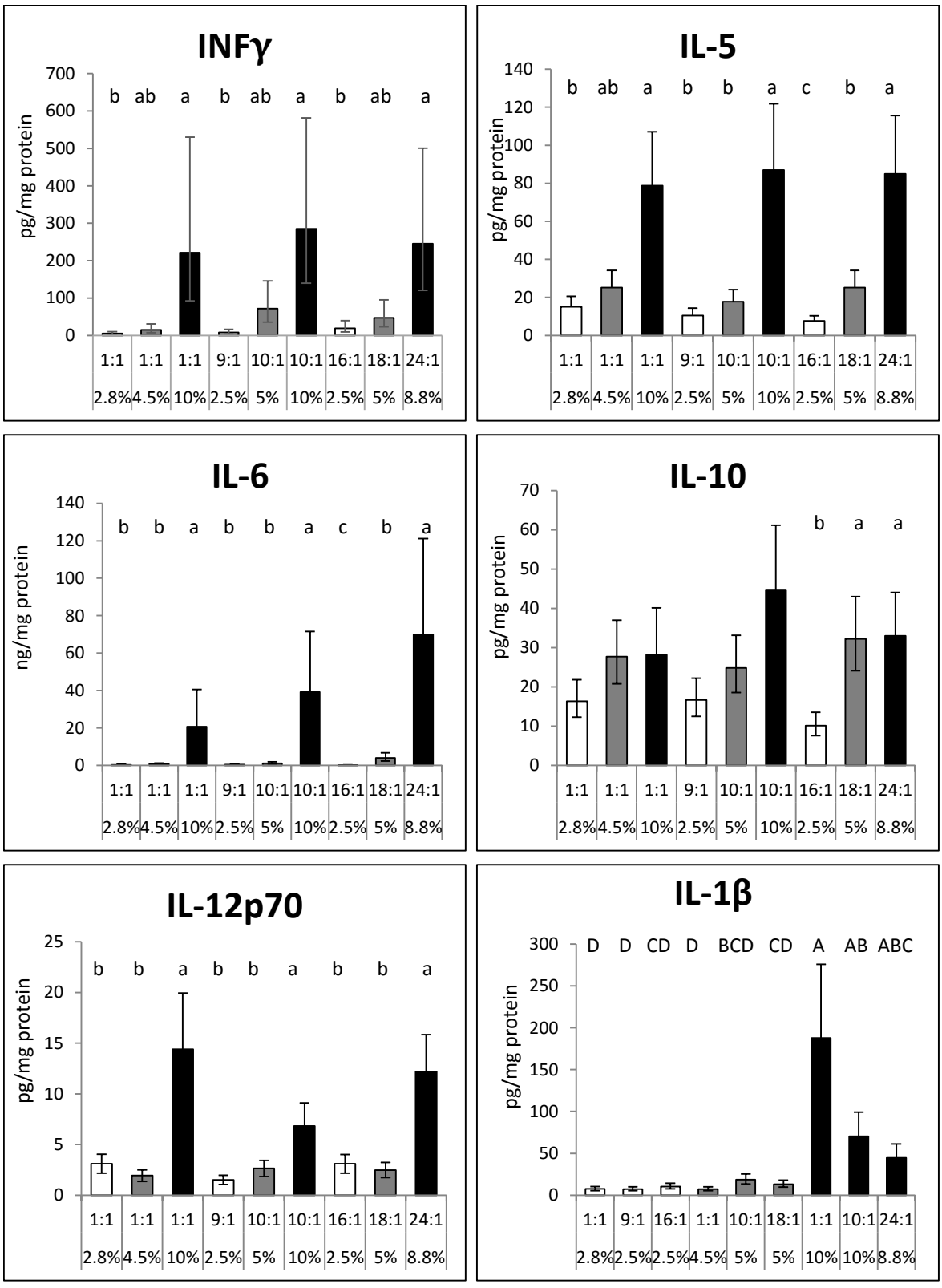
INFγ
pg/mg protein
700
600
500
400
300
200
100
0
b ab a b ab a b ab a
1:1 1:1 1:1 9:1 10:1 10:1 16:1 18:1 24:1
2.8% 4.5% 10% 2.5% 5% 10% 2.5% 5% 8.8%
IL-5
pg/mg protein
140
120
100
80
60
40
20
0
b ab a b b a c b a
1:1 1:1 1:1 9:1 10:1 10:1 16:1 18:1 24:1
2.8% 4.5% 10% 2.5% 5% 10% 2.5% 5% 8.8%
IL-6
ng/mg protein
140
120
100
80
60
40
20
0
b b a b b a c b a
1:1 1:1 1:1 9:1 10:1 10:1 16:1 18:1 24:1
2.8% 4.5% 10% 2.5% 5% 10% 2.5% 5% 8.8%
IL-10
pg/mg protein
70
60
50
40
30
20
10
0
b a a
1:1 1:1 1:1 9:1 10:1 10:1 16:1 18:1 24:1
2.8% 4.5% 10% 2.5% 5% 10% 2.5% 5% 8.8%
IL-12p70
pg/mg protein
25
20
15
10
5
0
b b a b b a b b a
1:1 1:1 1:1 9:1 10:1 10:1 16:1 18:1 24:1
2.8% 4.5% 10% 2.5% 5% 10% 2.5% 5% 8.8%
IL-1β
pg/mg protein
300
250
200
150
100
50
0
D D CD D BCD CD A AB ABC
1:1 9:1 16:1 1:1 10:1 18:1 1:1 10:1 24:1
2.8% 2.5% 2.5% 4.5% 5% 5% 10% 10% 8.8%

1
2

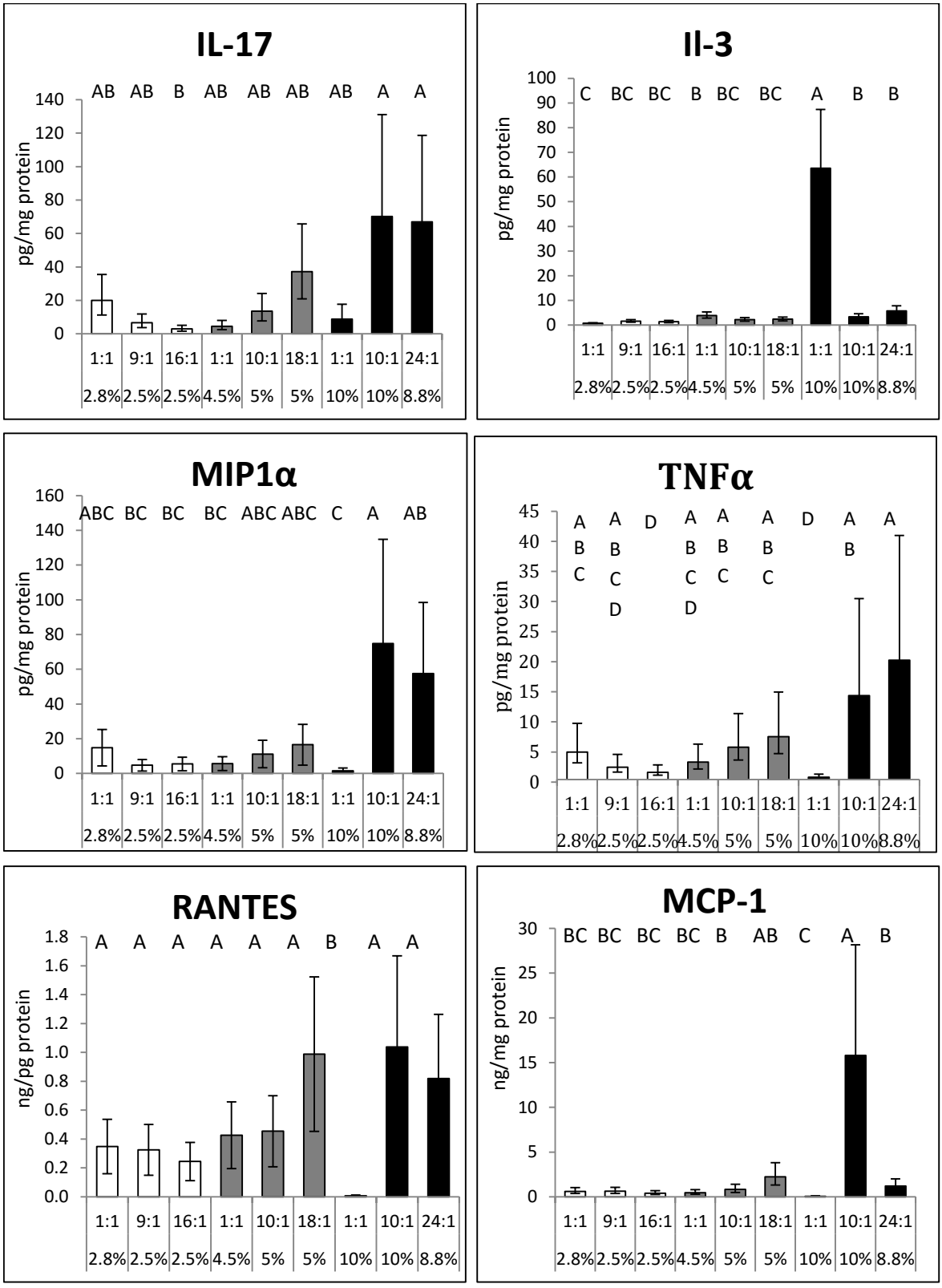
IL-17
pg/mg protein
AB AB B AB AB AB AB A A
1:1 9:1 16:1 1:1 10:1 18:1 1:1 10:1 24:1
2.8% 2.5% 2.5% 4.5% 5% 5% 10% 10% 8.8%
Il-3
pg/mg protein
C BC BC B BC BC A B B
1:1 9:1 16:1 1:1 10:1 18:1 1:1 10:1 24:1
2.8% 2.5% 2.5% 4.5% 5% 5% 10% 10% 8.8%
MIP1α
pg/mg protein
ABC BC BC BC ABC ABC C A AB
1:1 9:1 16:1 1:1 10:1 18:1 1:1 10:1 24:1
2.8% 2.5% 2.5% 4.5% 5% 5% 10% 10% 8.8%
TNFα
pg/mg protein
A B C A B C D D A B C D A B C A B C D A B
1:1 9:1 16:1 1:1 10:1 18:1 1:1 10:1 24:1
2.8% 2.5% 2.5% 4.5% 5% 5% 10% 10% 8.8%
RANTES
ng/pg protein
A A A A A A B A A
1:1 9:1 16:1 1:1 10:1 18:1 1:1 10:1 24:1
2.8% 2.5% 2.5% 4.5% 5% 5% 10% 10% 8.8%
MCP-1
ng/mg protein
BC BC BC BC B AB C A B
1:1 9:1 16:1 1:1 10:1 18:1 1:1 10:1 24:1
2.8% 2.5% 2.5% 4.5% 5% 5% 10% 10% 8.8%

Figure 2: Effect of diets on plasma cytokines 24h after acute LPS administration. Cytokines were analyzed using a two way ANOVA and significance differences further explored using the Tukey HSD test. For cytokines A-E there is an effect of PUFA, but no ratio effect nor an interaction. Lower case letters for these cytokines are only comparable within a PUFA level (2.5%, 5%, 10%). For cytokines F-L there was a PUFA × ratio interaction, and upper case letter indicate differences between treatments.