The Impact of Dietary Fat and Phosphatidylcholine on Increased Trimethylamine-N-oxide Levels.

By

Reem Ajlan

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Master of Life Science in Food Science & Technology

Andrew Neilson, Chair

Monica Ponder

Kevin Davy

Amanda Stewart

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Academic Abstract

Trimethylamine-N-oxide (TMAO) is an important biomarker of atherosclerosis. TMAO is the product of a hepatic conversion of trimethylamine (TMA). Releasing of TMA moieties is dependent on the adaptation of the gut microbiota to dietary TMA containing substrates such as phosphatidylcholine (PC), choline, and L-carnitine. A high-fat diet is an environmental risk factor that may increase TMAO production. However, it isn't clear if the high dietary intake of TMA is sufficient to promote increased plasma TMAO or if a high-fat intake is also required. We hypothesized that TMAO would be increased after consuming a high-fat diet and a high PC diet independently, with greater increases when consumed together. Four groups of twelve mice each were maintained on different treatments that were either low or high-fat with or without PC over two weeks. Then, a meal containing 9.99 g of corn oil and 0.75 g soybean L-α-Lecithin per 1 kg body weight was provided to all mice to indirectly observe the adaptation of the microbiota to the altered diet. The results of circulating TMAO levels showed that fat appeared to suppress TMAO production, which is against previous evidence. The microbial adaptation to the different treatments wasn't observed in the measurement of fecal TMA levels. As a result, our hypothesis was rejected. Future work addressing the impact of gene expressions of enzymes on the gut and the liver is needed. The use of another high TMA containing substrates such as choline and rats is recommended.

General Audience Abstract

Cardiovascular disease (CVD) is heart and blood vessel diseases - many of which are caused by atherosclerosis, a condition wherein fatty materials accumulate in the artery wall, reducing blood flow. The compound trimethylamine-N-oxide (TMAO) was found to be an important biomarker of atherosclerosis. TMAO levels increase in the body when gut microbiota releases trimethylamine (TMA) moieties from dietary phosphatidylcholine (PC), choline, and L-carnitine such as eggs and meat. A high-fat intake was believed to have an impact on increased levels of TMAO. However, it wasn't clear if the dietary intake of high TMA containing substrates such as PC, is sufficient to promote TMAO formation or if a high-fat content is also required. We hypothesized that TMAO would be increased after consuming a high-fat diet and a high PC diet independently, with greater increases when consumed together. The results would suggest new dietary strategies to avoid CVD. Four groups of twelve mice each were maintained on different treatments that were either low or high-fat with or without PC over two weeks. Then, a meal containing corn oil and PC was provided to all mice to observe the adaptation of the microbiota to the altered diet. The results showed that fat reduces circulating TMAO production, which is against previous evidence. Fecal TMA levels showed that microbiota activities weren't observed in the colon. As a results, no significant levels of TMA and its precursors were observed in feces.

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Chapter 1: Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide. Studies are moving forward to improve our understanding of the factors contributing to the incidence of this disease. Some of these factors are genetic sources, environmental sources, or a combination of genetic and environmental sources [4, 44]. Less than 20% of CVD risk is derived from genetic factors while environmental factors play a significant role in the development of CVD [76].

Previously, diets high in saturated fat such as meat were linked to CVD. Therefore, a reduction in dietary saturated fat has been believed to improve cardiovascular health. However, it was shown recently in a meta-analysis of prospective cohort studies that there is no strong evidence to confirm the hypothesis that the increase in saturated fat would increase the risk of CVD. That leads to the suggestion that there must be other factors or that the nutrients used to replace saturated fat are influencing CVD risk [17].

Trimethylamine-N-oxide (TMAO) is an important biomarker of atherosclerosis, a disease that narrows blood vessels through plaque build-up and leads to CVD [4,6,14]. The plaque is made of cholesterol, fat, calcium, fibrin, and other cellular waste products found in the blood [14,22]. Short-term high-fat diet was shown to increase postprandial TMAO concentrations in humans [1]. Therefore, a diet rich in fat is believed to be an environmental risk factor for the advancement of CVD [6]. Oral feeding of phosphatidylcholine (PC), choline, and betaine in mice has been shown to have strong associations with increased CVD risk [6]. PC, choline, and betaine are believed to be responsible, along with the contributions of gut microbiota effects, for the production of trimethylamine (TMA) that is converted by the liver enzyme into TMAO [6].

Hypothesis

Our central question is whether increases in dietary TMA precursors such PC are sufficient to induce increases of TMAO, or whether a high-fat intake is also required.

To determine if the intake of fat and PC increases the production of TMAO independently and/or synergistically, we hypothesized that TMAO would increase after consuming both a high-fat diet and a high PC diet independently, with greater increases when consumed together.

The aim

- Determine the impact of a high-fat intake and a high PC intake individually or together on TMAO levels.

To test the hypothesis, the following actions were done:

1- Forty-eight mice were maintained on special feeding - either a high-fat diet or a low-fat diet - with or without lecithin (PC) for 15 days.

2- At Day 16, mice remained on water only for twelve hours. Then, a high-fat high-PC meal was provided by gavage to all mice to measure fasting and postprandial TMAO levels. Blood drops were collected over 12 hours' post-gavage for TMAO quantification.

Chapter 2: Literature Review

Dietary Fat and Fat Digestion

Lipid digestion and absorption occur primarily in the stomach and the small intestine. Dietary lipids are triglycerides (90% of the total lipids), phospholipids, steroids (cholesterol that helps form cell walls, controls body fluids, and aids in the production of hormones [59]), fat-soluble vitamins (A, D, E, and K), and carotenoids. Lipid digestion enzymes are lipases (lingual, gastric, and pancreatic), cholesterol esterase, and phospholipases [72].

Lipases are involved in triglyceride digestion. Lipid digestion starts in the mouth wherein broken food is mixed with lingual lipase. This lipase catalyzes releasing of a short or medium chain fatty acid and 1,2 diglyceride. It is a very important enzyme for infants because of its ability to penetrate fat particles of milk releasing fatty acids since milk triglycerides are rich in short and medium chain fatty acids. It is stable in an acid environment, so it survives stomach acidity and acts as an aqueous environment leading lipids to coalesce and increasing their availability for enzymes [72]. In the stomach, gastric lipase catalyzes the hydrolysis of triglycerides into short and medium chains, some long chains fatty acids, and 1,2 diglycerides. The released short and medium chains and diglycerides incorporate into fat droplets providing a hydrophilic surface that influences the formation of an emulsion of lipids in the stomach. The emulsion is released as chyme to the duodenum [72]. In the duodenum, the chyme is combined with bile that is consisting of bile salt, phospholipids, and cholesterol. Bile acids are amphipathic that can further emulsify lipids, breaking lipid globule into small droplets, increasing the surface area for enzymes for further digestion. Free fatty acids, diglycerides, phospholipids, and bile salts together lead to the formation of micelles, which further increase the surface area for lipase activities to hydrolyze triglycerides. Triglycerides with short or medium chains can be cleaved

and absorbed without bile acids. In the jejunum, triglycerides digestion is continuous by pancreatic lipase, responsible for almost all fat metabolism, which catalyzes the cleavage of long fatty acids releasing free fatty acids and 2-monoglycerides. When 2-monoglycerides are absorbed by the small intestine, they undergo isomerization process in which the remaining fatty acids attach to carbon 1 or 3 [72]. In vivo, the pancreatic lipase needs to bind to a protein cofactor (colipase) for a greater hydrolytic process because lipid droplets are coated with phospholipids and bile acids that prevent the binding of lipase [72].

Cholesterol esterase activity is stimulated by bile salt. The enzyme is active on hydrolyzing ester bonds including the three ester bond (OH=O) in triglycerides, mono-glycerides, cholesterol esters, phospholipids, esters of vitamins A and D. Phospholipase is activated by trypsin in the intestinal lumen. The enzyme is capable of cleaving the two fatty acids in phospholipids that occur in micelles and lipid droplets [72].

The released fatty acids are a very dense source of energy. Fatty acids enter the citric acid cycle as acetyl-CoA to generate ATP (Adenosine triphosphate, a high-energy substance that stores the energy needed to carry out essential functions)[58,56]. They yield the most ATP compared to the other macronutrients when they are completely oxidized into CO_2 and water [58]. Excess lipid is stored as triglycerides in adipose tissues. The stored fat is essential in protecting internal organs and keeping body temperature [57]. Lipoprotein lipase cleaves the stored triglycerides to be used when it is needed [58].

Dietary Fat Influence the Development of Atherosclerosis

Though dietary fat is crucial for life, it influences human health in many ways including promotion of CVD. Consuming a high- fat diet decreases diversity in the gut microbiota [65] and

leads to unfavorable changes in the microbial population that contributes to the promotion of abnormalities in metabolism [21]. A high-fat diet was found to induce an increase in *Firmicutes sp.* and a decrease in *Bacteroidetes sp.*, both of the phylum are part of the normally obtained placental microbiome [65]. As a result, microbiota activates inflammatory pathways by manipulating lipid metabolism in adipocytes, macrophages, and vascular cells [21] and by forming bioactive compounds including secondary bile acids, short-chain fatty acids (SCFA), and TMAO inducing the development of atherosclerosis [21,54]. SCFA increase energy availability, and secondary bile acids increase energy expenditure. TMAO is associated with atherosclerosis via inhibiting reverse cholesterol transportation, inhibiting bile acids synthesis, promoting thrombogenic potential, increasing foam cell formation, and promoting endothelial dysfunction. On the other hand, a high-fat diet induces the liver to form more cholesterol [54]. Bile acids main functions are eliminating cholesterol from the body, facilitating absorption of lipid digestion products by emulsifying fat product into micelles, and aiding lipase digestive activity inside these micelles [52,55]. Because human bodies don't have enzymes to break down cholesterol, the inhibition of bile acids synthesis leads to elevated cholesterol level. HDL (high-density lipoprotein) and LDL (low-density lipoprotein) play an important role in transforming cholesterol back and forth between the liver and body parts. HDL, which is obtained from dietary fiber, fish, and omega 3 fatty acids, is responsible for collecting cholesterol and bringing it back to be excreted in the liver by bile acids. In contrast, LDL, which is obtained from a high-fat diet and a high-carb diet, inhibits of reverse cholesterol transportation [46]. Both of the lipoproteins are necessary, but it is important to keep HDL level high and LDL level low to avoid the accumulation of cholesterol in the arteries. Additionally, high-fat diet increases circulating lipopolysaccharide (LPS), that is released from bacterial wall inducing

atherosclerosis, by increasing intestinal bacterial translocation from colon as a result of reducing of tight junction proteins in the intestinal wall [65]. As a result of the reduction in bile acids synthesis, the inhibition of reverse cholesterol transport, and the induction of cholesterol synthesis, cholesterol and other fatty materials build up in the wall of the arteries causing atherosclerosis.

Saturated vs. Unsaturated fatty acids

Fatty acids are a diverse class of compounds, with different in chemical structures. Saturated fatty acids contain a chain of carbon atoms that connect to each other with single bonds, so each carbon atom bonds to two hydrogens, making the chain saturated with hydrogen. Unsaturated fatty acids contain double bonds between some carbon atoms. When a fatty acid has a single, double bond, it is called a monounsaturated fatty acid. When a fatty acid has two or more double bonds, it is called a polyunsaturated fatty acid [49]. The differences in chemical structures between fatty acids result in different physical properties. The chemical structure of unsaturated fatty acids gives them the flexibility to pass through the body. It is believed that the consumption of saturated fat results in elevated LDL level in plasma while consumption of unsaturated fat results in elevated HDL level [46].

Since the 1960s, many studies were performed addressing the link between saturated fat and heart and vessels diseases. As a result, a positive correlation between saturated fat and CVD was found [46]. However, in 2014, a meta-analysis of 72 studies suggested that there was no strong evidence to confirm the idea that saturated fat is linked to cardiovascular health or that polyunsaturated fatty acids can be protective [48]. The chair of the Nutrition Department at the Harvard School of Public Health, Walter Willett [48], criticized this paper, saying it "contains

multiple errors and omissions" and is "seriously misleading.[47]" The result of a study that was published in 2015 showed that the type of fat is critical. For 11 weeks, mice were fed either a high-lard diet or fish oil. The results showed that mice that consumed lard had developed signs of metabolic abnormalities while mice that consumed fish oil remained healthy. Also, mice fed lard showed increased levels of the microbial population such as *Bacteroides*, *Turicibacter*, and *Bilophila* while mice fed fish oil showed increased levels of the microbial population such as LAB, *Actinobacteria*, and *Verrucomicrobia*. When the gut microbiota was transplanted from the mice fed fish oil to antibiotic-treated mice, and after providing a lard-diet, the researchers found that these mice have remained healthy [50,51]. The results of this study demonstrated that the composition of gut microbiota differed when different fats were consumed, and some microbial population was protective against unhealthy effects of saturated fatty acids [50]. Despite the fact that it isn't clear if saturated fat has a direct correlation with cardiovascular disease, high intake of unsaturated fat can cause as much harm as consuming saturated fat. In this paper, we focused on the presence of fat rather than the type of fat.

What Is TMAO?

TMAO is a small organic component that is derived from TMA and is present in the blood in high concentrations after ingesting dietary choline, L-carnitine, and phospholipids [5]. It belongs to an amine oxides class, and it has the formula $(CH_3)_3$ NO [5], see **Figure 2.1**. Importantly, TMAO is present in high concentrations in marine animals [5]. Therefore, consuming seafood increases TMAO concentrations in plasma [5]. The results of a recent study showed that the consumption of seafood (fish) led to higher increases in circulating TMAO and urinary concentrations of TMAO than the consumption of dietary choline (eggs) and L-carnitine (beef)

in humans. The consumption of fish elevated circulating TMAO levels within 15 min. This finding led to suggest that TMAO might be absorbed directly from the gastrointestinal tract without undergoing gut microbiota contribution and present at high levels in plasma [66].

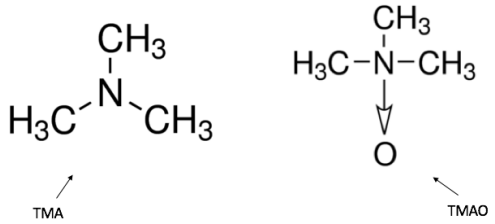

Figure 2.1 The chemical structure of TMA and TMAO.

TMAO concentration in blood is in the range of 0.5 - 3 μmol/L in healthy people up to 40 μmol/L in patients with kidney failure. However, TMAO concentration in rats is approximately 0.6 μmol/L and less than five μmol/L in mice. The different levels of TMAO between human being, rats, and mice are dependent on many factors, including the amounts of dietary carnitine and choline, the action of gut microbiota to free the TMA moieties, and the flavin monooxygenase 3 (FMO3) activity [5].

Production of TMAO

TMAO is the product of the consumption of dietary substrates containing TMA moieties such as choline, L-carnitine, and phospholipids. Gut microbiota is responsible for releasing TMA from dietary substrates choline, L-carnitine, and phospholipids such as PC. The microbiota uses the enzymes phospholipases and glycerophosphoryldiester phosphodiesterases to release choline from PC. Then, microbiota uses the enzyme TMA lyase to cleave the C-N bond [4] releasing TMA moieties from choline [1,5]. TMA lyase that releases TMA moieties from choline was found with

choline utilization gene cluster (cut) that contains (catalytic and regulatory protein cutC and cutD, both of which are required for releasing TMA from choline in an anaerobic condition). TMA lyase that releases TMA moieties from L-carnitine was found with L-carnitine utilization gene cluster that contains (catalytic and regulatory protein CntA and CntB). Quantitatively, it is not known how these microbial enzyme clusters are related to TMA and TMAO production [4]. The free TMA is oxidized in the liver into TMAO by a hepatic enzyme called flavin monooxygenase 3 (FMO3) [1,5]. TMAO accumulates in the tissues as osmolyte or is cleared by the kidneys as a waste product [70], see **Figure 2.2**. Based on this finding, we hypothesized that the diet, microbiota, and genetic factors are a perfect combination that leads to atherosclerosis and CVD [4,15].

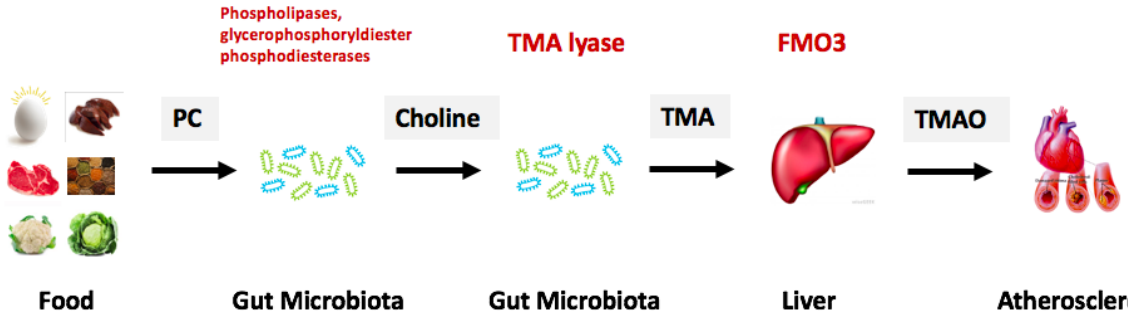

Figure 2.2 Illustration of TMAO formation as a result of consuming a diet high in TMA precursor, PC.

The Role of Gut Microbiota

The human gut shelters more than 100 trillion healthy microbial cells [4]. However, diet manipulates the composition of gut microbiota in humans [23]. The results of using 200 strains of mice showed that variations in gut microbiota are due to dietary factors, not genetic factors [65]. A microbial population can change incredibly fast in the average of three days when they experience a significant shift in dietary intake [62]. Changes in the composition of microbiota

affect the entire immune system which then leads to many diseases [28]. Gut microbiota as an environmental factor has been related to intestinal health, immune system efficiency, and nutrients and vitamins bio-activation. Recently, it has been linked to obesity and insulin resistance which both are associated with CVD [18,19,20]. They are associated with obesity due to an elevation in microbial capability to metabolize undigested food components (fiber) into SCFA that result in increased lipid load in adipocytes [54, 65].

Microbiota modulates several signaling pathways in the gut such as inflammation and lipid metabolism that is associated specifically with atherosclerosis [21]. The microbiota is essentially an endocrine organ that metabolizes nutrients that depend on microbiota for metabolism producing hormonal agents such as TMAO, SCFA, and secondary bile acids [76].

Gut microbiota elevates circulating levels of TMAO as a result of consuming choline or L-carnitine continuously [54]. Some species of gut microbiota were found to prefer these nutrients [54] and depend on them as a fuel source releasing more TMA [1]. To test whether microbiota is essential in TMAO production, subjects were given dietary L-carnitine plus L-carnitine supplements. The same subjects were treated with antibiotics to eliminate microbiota. By measuring endogenous TMAO production from this nutrient, the results showed that production of TMAO is dependent on the contribution of gut microbiota [12]. Because of the diet-induced CVD risk, gut microbiota proved to be an environmental risk factor for heart diseases [3,6]. A study by Koeth *et al.* was designed using both healthy patients and mouse models. The data showed that gut microbiota adapted to animal-based diets and produced TMA. The result illustrated that elevated levels of TMA and TMAO are a direct cause of atherosclerosis in mice, but are associated with CVD in humans [12].

Identifying bacteria that produce TMAO and is associated with CVD may lead to possible suggestions to avoid this disease [20]. After an L-carnitine challenge, Koeth et al. found a positive association between specific bacterial taxa, belonging to the families *Clostridiceae* and *Peptostreptococcaceae*, in human feces and high concentrations of plasma TMAO, based on animal-based diet [12]. This finding leads to suggest that L-carnitine could be metabolized by bacteria belonging to these families [20]. Several species were also identified in TMA and TMAO production in humans and animals belonging to phylum *Firmicutes*, *Proteobacteria (Enterobacteriaceae)*, *Actinobacteria*, *Bacteroidetes (Prevotellaceae)*, *Deferribacteres (Deferribacteraceae)* and *Tenericutes (Anaeroplasmataceae)* [54,73]. In a new study, researchers were able to identify nine stains capable of releasing TMA from choline in vitro from human intestine. Eight species belong to the phyla *Firmicutes* or *Proteobacteria.* Six genera showed significant TMA production from choline including *Anaerococcus hydrogenalis*, *Clostridium asparagiforme*, *C. sporogenes*, *C. hathewayi*, *Proteus penneri*, *Escherichia fergusonii*, *Providencia rettgeri*, and *Edwardsiella tarda* [70]. Recent studies have failed to identify common genera that associated with plasma TMAO levels in humans and mice [12].

Some anaerobic microbiota located in the cecum and colon are capable of producing SCFAs by fermenting non-digestible carbohydrates. SCFAs, recognized by G protein-coupled receptors (GPR41and GPR43), regulate several signaling pathways such as energy metabolism, insulin sensitivity, and immune cell programs and receptors involved in CVD via increasing energy availability [76].

Unabsorbed bile acids move to the colon wherein they are converted into secondary bile acids (removal of the glycine and taurine groups) by facultative and anaerobic bacteria. Secondary bile acids, recognized by the nuclear receptor farnesoid x receptor (FXR) and a G

protein-coupled receptor (TGR5), regulate several signaling pathways including energy expenditure, cholesterol balance, and insulin sensitivity [76].

Additional to the association of the microbial-derived metabolites to CVD, gut microbiota release compounds from their cell wall including LPS and peptidoglycan that are associated with CVD as well [76].

Microbiota species such as *Firmicutes sp.* and *Bacteroidetes sp.* release LPS that are carried by chylomicrons via intestinal tight junction barrier into circulation. The released LPS and free fatty acids act synergistically in the promotion of adipose inflammation and foam cell formation. Toll-like receptor 4 (TLR4) recognizes LPS in macrophages [54]. As a result, it down-regulates expression of liver X receptor (LXR) and up-regulates expression of LDL, very low-density lipoprotein (VLDL), and adiponectin receptor 2 (AdipoR2), which is involved in fatty acids breakdown. LXR regulates cholesterol balance in macrophages, liver, and small intestine by inducing reverse cholesterol transportation (RCT) and promoting degradation of receptors related to lipid metabolism. LPS activity leads to increasing the uptake of lipid by macrophages elevating their transformation to foam cells. Additionally, LPS lowers the formation of glucagon-like peptide 2(GLP2) intestinal neuroendocrine L-cells which as a result weakens of colon epithelial integrity and elevates extra-intestinal efflux of colon biomaterials all of which result in enhanced inflammation, see **Figure 2.3**. A relatively low dose of LPS can cause many pathogenic effects to macrophages. LPS absorption by the intestine can be suppressed via the inhibition of chylomicrons [54]. Treating mice with antibiotics or plants such as fermented green tea reduced of LPS levels and inflammation [65]. Additionally, peptidoglycan that is recognized by nucleotide oligomerization domain-containing receptors (NODs) manipulates lipid metabolism and activates macrophages promoting inflammatory responses [76].

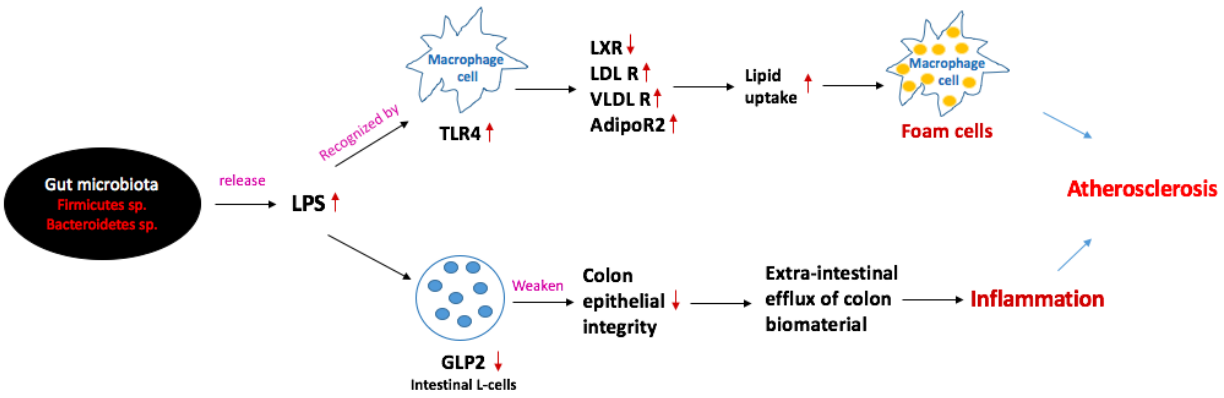

Figure 2.3 Microbiota contribution to the development of atherosclerosis through lipopolysaccharides [54].

Hepatic FMO3

FMO3 is one member of a large enzyme family called flavin-containing monooxygenases (FMOs). FMO3 that is made in the liver is an enzymatic catalyst for production TMAO in people. Their role as enzymes is breaking down compounds that contain nitrogen (such as TMA), sulfur, and phosphorus [16]. After examining the activity of FMO family members, it was found that FMO1 and FMO3 are the only two enzymes that synthesize TMAO significantly. However, FMO3 was shown to be the most active enzyme, responsible for more than 90% of the total hepatic activity, compared to FMO1 and the other FMO family members in metabolizing nitrogen-containing compounds derived from the diet such as TMA. As a result, an increase in hepatic expression of the FMO3 protein in vivo will increase TMAO production in plasma [24]. As previously mentioned, it oxidizes TMA, which is the molecule that gives fish their fishy smell, in the liver and converts it into TMAO, which has no odor. TMAO leaves the body through urine. A positive association between TMAO levels and FMO3 has been noted after examining

samples from liver and plasma in humans [5,16]. A mutation of FMO3 results in excreting of TMA rather than TMAO. This situation is known as trimethylaminuria (fish odor syndrome) [70].

Upregulation of hepatic FMO3 was found to be dramatically controlled by bile acids by the action of FXR [4] (NR1H4), and that leads to an increase in the synthesis of TMAO. In contrast, it was found that FMO3 is downregulated by testosterone in mice, a hormone that is responsible for male sexual characteristics [24,25]. This finding explains why the hepatic FMO3 expression is reduced in males compared to females in both humans and mice. As a result, female mice were found to have greater susceptibility to atherosclerosis compared to males, as well as in human beings. The difference in FMO3 levels between males and females, approximately 100-fold, was found to be limited to liver and adrenals, not involving the aorta and lungs [24]. However, the FMO3 function is reduced during menstruation [64].

Further studies were conducted searching for other factors that contribute to an increase in TMAO production in female compared to male mice. First, the effects of a choline-enriched diet on the expression of hepatic FMO3, enzymatic activity, and plasma of TMA and TMAO were examined in both genders of mice. As a result, the diet with choline supplementation (1%) didn't affect hepatic FMO3 gene expression in both genders. However, choline supplementation caused a large increase in TMAO levels in both genders. This increase was higher in female mice compared to males due to the higher hepatic FMO3 expression in female mice. Conversely, TMA levels in male mice were higher than female mice as a result of a reduction of the synthesis of TMAO. These results weren't due to altered FMO activity. Although the difference between genders was expected, the ability of FMO3 to produce TMAO was independent of the diet [24].

The results of another study, after isolating gut flora from different intestinal regions in both genders in mice, suggesting that the difference in TMAO levels and FMO3 expression

between the two genders wasn't due to the action of gut microbiota [24]. We conclude that both diet and gut flora have major effects on TMAO production, but they aren't responsible for the difference in TMAO levels between genders. Also, the effects of gender on FMO3 expression is exclusively due to a hormonal effect, not to the alteration of FMO3 [24].

Phosphatidylcholine (PC)

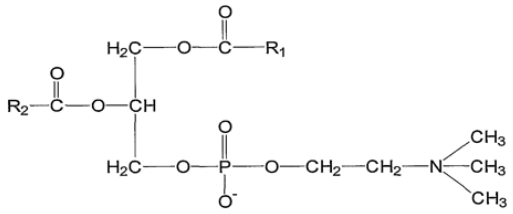

Figure 2.4 A general chemical structure of phosphatidylcholine - lecithin.

Since this study addresses PC (an extraction form of PC from food called lecithin. Lecithin consists of different PCs, but not all PCs are lecithin) as a potential factor that increases TMAO concentration in plasma, it is important to know some facts about it. PC is a phospholipid that is made of two fatty acids (usually one of them is saturated and the another is unsaturated) and a phosphate group binds a hydrophilic group (choline) attached to the glycerol backbone, see **Figure 2.4**. PC is amphipathic; it has one hydrophilic end and another hydrophobic end, which allows it to make up the outer cell membranes [45]. It is a major dietary source of choline. Therefore, PC digestion increases the level of choline and betaine in plasma [4], as well as, the major fate of all choline consumed by humans that it is immediately made into PC, which is then integrated into cell membranes, see **Figure 2.5**. Most phospholipids that occur in most mammalian membranes are PC. It also occurs in all nucleated cells [29,30].

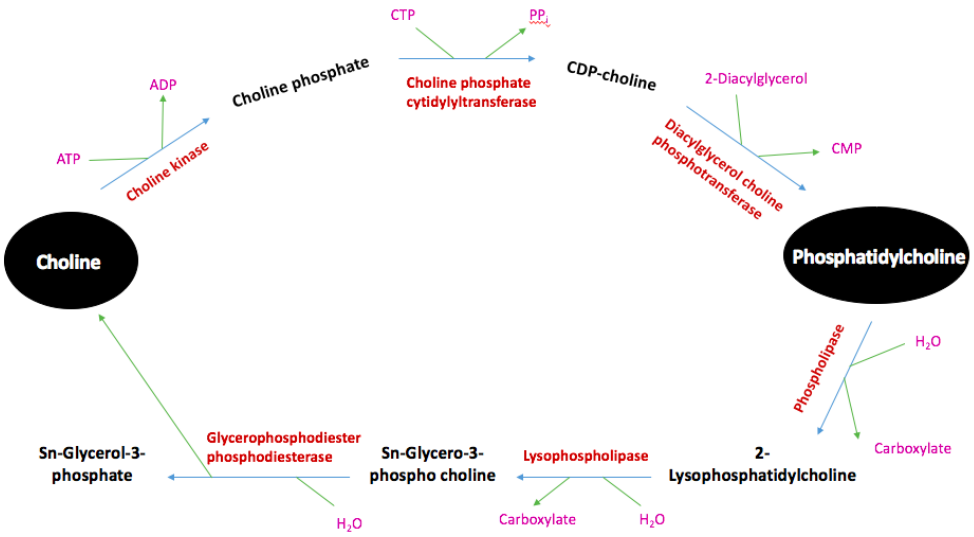

Figure 2.5 Phosphatidylcholine and choline metabolism [60].

Choline role in the body is poorly understood. It has been shown that choline has a lot of benefits that enhance human health. As previously mentioned, it is required to make phospholipids that are essential components of all membranes. Choline is also a part of the folate cycle that is important for liver, brain, and nerve health. It is required to form VLDL particles that carry triglycerides out from the liver into the bloodstream. The absence of PC or choline results in a fatty liver disease because of the accumulation of triglycerides inside the liver. It also has been shown that PC improves our memories, intestinal, and skin health, maintains cell structure, and metabolizes fat [34].

Therefore, choline deficiency is a real problem because it will affect not only the liver but also cell membranes such as nerve cells. The impact of choline deficiency was shown to extend to CVD, atherosclerosis, and neurological disorders [29,30]. In fact, low consumption of choline was linked to increased levels of homocysteine in plasma as a result of the reduced capacity of converting it to methionine. High levels of homocysteine in plasma have been associated with

several chronic diseases such as CVD, cancer, and bone fracture [29,38,39,40]. In contrast, increasing the intake of choline and betaine was found to decrease levels of homocysteine in plasma. After treating a group of men, aged 50-71, that were diagnosed with high levels of homocysteine with a high daily dose of choline and then applying a methionine loading test, Olthof et al. found that fasting, as well as plasma homocysteine concentrations, were lowered. In the results from this study, Olthof et al. suggested that if the intake of choline was capable of lowering the elevated homocysteine, the intake of choline might reduce the risk of CVD [29]. It should be mentioned that homocysteine levels can be lowered to form methionine via another pathway involving vitamin B12 and folic acid [29]. Recently, it was found that when choline supply is low, it is recycled in the liver. Also, it is redistributed from the kidney, lung, and intestine to be reused in the liver [29,37].

The requirement of choline is dependent on the combination of a person's diet and genes. It has been shown that the more energy such as sugar and fat, delivered to the liver, the more the choline will be needed to remove the accumulation of fat that results from this delivery [32]. In addition to that, the ability of people to make choline is dependent on genetic factors. For example, it was suggested that Asians have a stronger ability to make choline from the amino acid methionine than Caucasians [32]. PC levels have been shown to decrease as we age, especially in brain tissue [34], so the demand for PC increases with age.

The recommendation of adequate intake for choline has been set at 420 mg/d for women to 450 mg/d for lactating women and men, but the lack of human data is a limitation in determining the Estimated Average Requirement [29]. Some researchers argued that most people aren't getting enough choline from a modern diet which is as a result is responsible for a silent epidemic of fatty liver disease [33]. The human daily intake of PC is approximately two to eight g

per day which is low [68]. However, the bile synthesizes 10-20 g per day of phospholipids, mostly PC [72].

Choline can be found mostly in organ meats such as liver and eggs [33]. It can also be found in plant-based foods such as whole grains, soy, and vegetables such as cauliflower and cabbage [5]. However, the amount of choline in liver and eggs is ten times greater than most plants [33]. Therefore, people who consume animal-based high-fat foods are believed to be more likely to have high concentrations of TMAO in plasma [1,5,6]. The intake of PC supplements was pointed to be important during specific timing such as pregnancy and lactation [29]. The findings of many studies suggested that PC supplements can enhance learning, improve memory, promote skin health, and help liver function [29]. Also, some dietary supplements with high concentrations of PC are shown to improve heart and liver function such as those which are marketed as anti-dementia drugs or products [5].

After all of these facts about PC, is it possible to say that it is bad for our health? Though choline has been found to protect us against fatty liver disease, that is, an independent risk factor for heart disease [31], how is it possible that choline is responsible for the development of atherosclerosis?

PC was found to be a potential risk factor for atherosclerosis. A group of researchers found a strong association between the consumption of PC or its metabolites and CVD risk in mice [6]. In a new study, the same research group applied a phosphatidylcholine challenge on healthy human participants; measurements were taken before and after providing antibiotics to inhibit gut microbiota. As a result, TMAO and other choline metabolites levels were detected after the challenge. TMAO levels in plasma were inhibited after the exposure to the antibiotics. In the same study, the relationship between TMAO levels and incident adverse cardiovascular

events was examined in 2,007 patients through three years of follow-up. As a result, there was a positive association between high levels of TMAO in plasma and the risk of major adverse cardiovascular events. That means that the presence of TMAO levels in fasting plasma is either a predictor or causative of cardiovascular disease events [15].

However, Masterjohn in his 2010 paper argued that human studies have not shown any detectable increases in TMA levels after consuming either phosphatidylcholine or choline-rich foods [31]. By including a result from an old study on PC, he argued that consuming PC by humans led to a small increase in TMA only. This increase was even a result of consuming lecithin that was contaminated with TMA, so cleaning up lecithin from this contamination, by removing TMA, didn't increase urine extraction of TMA [31,35]. Additionally, after providing either choline or betaine diet to women aged 49-70, it has been found that neither the intake of dietary choline nor betaine were associated with CVD risk [29,41]. The results from another study led to the same conclusion. Forty-six different foods were provided to humans. By looking at the urinary excretion of TMA and TMAO in humans, the authors found that 60% of free choline and 30% carnitine were converted to one of these two products, but they didn't see any levels of these two products in the urinary excretion after consuming either PC or betaine [31,36]. Masterjohn suggested that neither choline-rich foods such as eggs and liver nor carnitine-rich meats produced TMA or TMAO over control levels. The only foods that were found to increase the urinary extraction of these two products were seafood [31,36]. The result from this study suggests that even if we consume meals high in these products, our kidneys excrete them very well. Also, there is no strong evidence to support the hypothesis that consuming seafood is associated with increased CVD risk. Instead, it is known that islanders who consume seafood very often are less likely to have CVD risk [31,36].

To overcome this problem, humans should probably limit the intake of PC and its metabolites. However, decreasing this intake has been found to cause fatty liver and other serious diseases. Therefore, it would be important to inhibit the gut microbiota since its role is essential in the production of TMA. Also, if commercial PC was found to be truly contaminated with TMA, it should be cleaned before using it in future studies. If consuming food rich in high TMA containing nutrients such as choline over control levels, doesn't produce TMA and TMAO. If human kidneys were found to excrete TMA and TMAO very well and if the intake of PC metabolites has no direct association with CVD risk in humans, is increasing the intake of PC a problem [12,31]? The answers to these questions are needed to improve our understanding of the consequences of consuming PC and its metabolites.

TMAO and Atherosclerosis

TMAO and its precursors including PC, choline, and betaine are highly associated with atherosclerosis and CVD risk. TMAO levels have shown positive correlations with plaque size in the artery of CVD patients. In human studies, TMAO was an indicator for major adverse cardiac events (MACE) such as stroke [71]. The mechanism by which TMAO is linked to the development of atherosclerosis is unclear [12]. Recent results suggested that TMAO leads to the development of atherosclerosis by manipulating the metabolism of cholesterol and sterol. A study demonstrated that TMAO, choline, and carnitine play important roles in the inhibition of the reverse cholesterol transportation through gut microbiota-dependent mechanisms [5]. In addition to the inhibition of reverse cholesterol transportation, recent evidence suggested that TMAO reduces bile acid synthesis from cholesterol and decreases bile acids transporters expression in the liver [54], both of which are linked to increased atherosclerosis [20]. TMAO promotes inhibition of

expression of a hepatic gene Cyp7al that catalyzes bile acid synthesis and a rate-limiting step in cholesterol catabolism [12]. It is still unclear how TMAO affects these pathways, and whether this mechanism may occur by binding between TMAO and a receptor, or whether TMAO has an impact on enzymes directly [20]. The available results also suggested that TMAO enhances thrombogenic potential [43] and promotes foam cell formation [6] which as a result cause atherosclerosis. TMAO alters stimulus-dependent calcium release on platelet enhancing platelet aggregation responses to multiple agonists including thrombin, adenosine diphosphate, or collagen [71]. TMAO induces cholesterol uptake by macrophages through activating expression of proatherogenic scavenger receptors SRA and CD36 leading to the formation of foam cells [54]. Additionally, TMAO is associated with aging-associated endothelial dysfunction by reducing the bioavailability of nitric oxide (NO)[74] and increasing leukocyte adhesion that is mediated by nuclear factor-κB (NF-κB) signaling pathway [75]. Increasing vascular inflammation and oxidative stress weaken of endothelial nitric oxide synthase (eNOS), that produces NO, resulting in an imbalance between vasoconstriction and vasodilating endothelium substances [74]. Still, TMAO receptors are unknown [4]. It was suggested that TMAO that act as a small chaperone is directly capable of inducing changes to protein structure including protein folding stability and functionary. As a result, TMAO doesn't need a receptor to be able to alter of signaling pathway [12].

According to a study, increasing the choline in a diet for a mouse that was liable to develop atherosclerosis not only resulted in increased TMAO, but also to a significant development of plaque in the arteries. The result of this study demonstrated that patients with highest TMAO concentration had a higher risk of MACE [8] compared to the other patients with lower concentrations [3]. However, not all human studies have shown positive correlations

between TMAO and atherosclerosis. In some studies, the consumption of meat, fish, and egg wasn't associated with increased TMAO, choline, or betaine levels [70]. Some studies suggested that TMAO might protect the cardiovascular system by decreasing the bad effects of oxidative stress [5].

TMAO Promotes the Increase of Other Diseases

TMAO and chronic kidney disease (CKD):

After the contribution of both gut microbiota and FMO3 to produce TMAO, TMAO moves to the kidneys where it promotes renal injury, renal fibrosis, and renal dysfunction. Available evidence suggested that TMAO increases renal fibrosis and renal dysfunction by promoting phosphorylation of Smad3 that regulates the pro-fibrotic transformation growth factor TGF-β/ Smad3 signaling pathway. Based on this evidence, TMAO was shown to be not only a biomarker but also associated with the progression of renal diseases [7]. The results of mouse studies that were fed either 0.12% TMAO or 1% choline with standard mouse chow for six weeks showed a massive increase in TMAO levels, as were seen in people with CKD. In the same study, extending the feeding time with choline or TMAO for 16 weeks resulted in a significant increase in the levels of serum cystatin C, a protein that is used mostly as a biomarker of renal function [7,26]. To get a better understanding of the contribution of TMAO to CKD in humans, a recent study of three groups that were different in health status including a control group, CKD3-4 stages, and CKD 5 stage was applied. The results showed that patients with CKD 5 to have the highest TMAO concentrations. Also, TMAO levels were shown to be responsible for the increase in inflammatory biomarkers. Furthermore, the effects of dialysis, a treatment that mimics kidney function when kidney reaches 85-90% failure [27], and renal

transplantation (Rtx) on high TMAO concentrations were studied. As a result of 12 months of dialysis, betaine and choline increased, but TMAO levels remained the same. However, TMAO levels dramatically decreased after Rtx. In contrast, choline and betaine concentrations increased. Also, it was seen that TMAO levels were indicators for mortality of patients with CKD 3-4 stages [10]. Some human studies haven't shown significant associations between TMAO levels and CKD. Still, it is unclear whether TMAO levels in CKD are elevated as a result of increased formation or decreased elimination or whether TMA is used as an alternative for other retention solutes [70].

Therapeutic Strategies to Reduce TMAO Production

A lot of evidence has shown the role of gut microbiota in manipulating human health causing metabolic disorder, inflammation, and atherosclerosis. The interaction of gut microbiota with diet and host genetics lead to the production of TMAO that is associated with CVD. Therefore, some researchers have studied the possibility of modulating the microbiota composition via treatment with antibiotics, probiotics, and prebiotics [70]. Oral intake of antibiotics decreased circulating TMAO levels in healthy subjects after a PC challenge. The antibiotic effect has stopped one month after stopping using them. Treating with an antibiotic is not ideal to suppress TMAO production because chronic treatment with antibiotics could lead to producing resistant bacteria and other undesired consequences such as reducing the number of beneficial microbiomes [70]. Probiotics increase the diversity of gut microbiota that could provide a healthy microbial environment. Treatment with probiotics such as VSL#3 has been shown to inhibit the harmful effect of gut microbiota resulted in significant decreases in TMAO concentrations in the blood of humans and laboratory animals, which led to the prevention of atherosclerosis [3,5,9]. In

another study, treatment with another probiotic didn't reduce TMAO levels in plasma and didn't affect plasma concentrations of L-carnitine, choline, or betaine [2]. Additionally, treatment with prebiotics such as resveratrol (RSV), that naturally occur in grapes and berries, increases in *Bacteroidetes*, a phylum that contributes very little to the formation of TMA[73], over *Firmicutes*, a phylum that contributes more often with the formation of TMA[73], in mice treated with choline. TMA and TMAO serum levels were much lower in treated mice than in control mice [70]. A possible treatment for TMAO-associated CVD events is reducing the intake of dietary precursors of TMAO including choline, PC, and L-carnitine. However, choline is an essential nutrient of which its deficiency causes serious diseases such as fatty liver. Another suggestion would be switching diet habits such as being vegetarians instead of omnivore since the amount of TMAO precursors are much higher in animal-based diets compared to plant-based diets. Reducing the fat intake is associated with decreasing TMAO levels. A high-fat diet provides the body with dietary cholesterol and phospholipids. TMAO precursors are more likely found in a high-fat diet such as meat and eggs. Additionally, a high-fat diet increase of microbiota associated with TMA formation, such as *Firmicutes.* Therefore, limiting the intake of fat might be associated with reducing the risk of increased TMAO levels. We have evidence illustrating the positive association between circulating TMAO levels and the consumption of marine animal such as fish [66]. However, fish consumption is inversely associated with chronic heart disease [54]. Inhibition of the enzyme FMO3 activity is a potential approach to reduce the conversion of TMA to TMAO. A known consequence of altered FMO3 is trimethylaminuria, which is also known as a fishy odor syndrome [70]. An ideal treatment for TMAO production would be an inhibitor of microbial TMA lyase. For example, 3,3-dimethyl-1-butanol (DMB), which is found in some balsamic vinegar and olive oils, is a structural analog of choline that is capable of inhibiting TMA

formation by the inhibition of microbial TMA lyase without altering cholesterol levels. DMB suppressed choline metabolism and promoted a reduction in microbiota associated with circulating TMA and TMAO levels in mice [70]. Treatment with DMB could be a future strategy to avoid TMAO production in humans as well. Meldonium, a structural aza-analog of gamma-butyrobetaine, was found to compete with L-carnitine and gamma-butyrobetaine for transporters and enzymes such as TMA lyase decreasing TMA and TMAO levels. However, it doesn't influence the microbial size and dietary uptake of L-carnitine [70]. Future studies are needed to suggest new efficient strategies, protective therapies or supplementation that are safe for human consumption to avoid TMAO accumulation in plasma [71].

Conclusion

TMAO and its precursors are highly associated with atherosclerosis. TMAO is produced after the consumption of dietary TMA containing substrates such as L-carnitine, PC, and choline Gut microbiota uses the enzyme TMA lyase to cleave the C-N bond from choline and L-carnitine releasing free TMA moieties. The free TMA is oxidized by hepatic FMO3 into TMAO [6,15]. TMAO levels in plasma were inhibited after the exposure to antibiotics leading to suggest that TMAO production is dependent on the microbiota contribution [12]. However, the consumption of fish elevated circulating TMAO levels in the body within 15 min as a result of direct absorption of TMAO from the gastrointestinal tract [66]. PC as a potential factor for atherosclerosis has a complex role in human bodies. It is an essential component of all membranes. It is a primary dietary source of choline that enhances human health in several ways. The absence of choline might result in many serious diseases such as a fatty liver. The impact of choline deficiency was shown to extend to CVD, atherosclerosis, and neurological disorders [29,30]. On the other hand, the

results of recent studies showed a strong association between the consumption of PC or its metabolites and CVD risk in mice and humans. A diet rich in fat is believed to be an environmental risk factor for CVD. However, it is still unclear if the dietary intake of high TMA containing substrates such as PC, is sufficient to promote TMAO formation or if a high-fat intake is also required.

The mechanism by which TMAO is associated with the development of atherosclerosis is unclear. Available evidence suggested that TMAO inhibits of reverse cholesterol transportation, reduces of bile acid synthesis from cholesterol, inhibits expression of bile acids transporters in the liver, [20] induces cholesterol uptake by macrophages leading to foam cell formation, promotes endothelial dysfunction, and promotes a thrombogenic potential [54]. However, TMAO receptors are still unknown.

Recent studies have suggested therapeutic strategies to avoid increased circulating TMAO levels. The use of antibiotics has shown to decrease TMAO levels. However, chronic antibiotics intake might lead to selection for resistant bacteria. Treatment with some probiotics and prebiotics have altered in microbial population lowering TMAO levels. Reducing the uptake of TMAO precursors by reducing the intake of a high-fat and an animal-based diet was considered. However, reducing the intake of choline and PC was associated with several health problems. Additionally, the inhibition of FMO3 expression was found to reduce TMAO production causing the body to excrete TMA instead of TMAO (fishy odor syndrome). Finally, treatment with Meldonium and DMB targeting microbial TMA lyase and its actions resulted in lowering of TMA formation in mice [70]. Further studies are needed to test these compounds on humans.

Chapter 3: Research

3.1 Objectives

Previous studies have used either a high-fat diet with dietary TMA precursor supplementation or a diet high in both fat and TMA precursors. However, it is believed that no studies had been performed addressing different dietary patterns. The main aim was performing a study that was designed specifically to show how manipulating diet compositions (intake of fat and PC) increases circulating TMAO and fecal TMA levels in mice. We hypothesized that plasma TMAO would increase after consuming both a high-fat diet and a high PC diet independently or synergistically, with greater increases when consumed together. Four groups of 12 mice each were maintained for two weeks on either a high-fat or a low-fat diet with or without PC. It is believed that dietary intake of TMA containing substrates changes gut microbiota dietary preferences altering their communities after the consumption for an average of three days. That leads to increased levels of released TMA by the gut. However, it was unclear by which dietary pattern the microbiota is capable of releasing more TMA moieties. Therefore, an acute meal that was made of corn oil and PC was provided to all treatments at the end of the study, and blood drops were collected over 12 hours. We indirectly measured gut bacterial adaptation to the altered diet through measurement of fasting and postprandial TMAO levels, fecal TMA levels, and non-fasting plasma TMAO levels. If adaptation occurred, this leads to increase in released TMA by the gut and may lead to increase in hepatic TMA conversion of TMAO.

3.2 Materials and Methods

3.2.1 Quantification of Phospholipids

Introduction

Before performing the animal study, it was essential to know which fat could be used in the diets without supplying significant TMA moieties. We wished to use exogenously added PC in the diets, so we didn't want other phospholipids to interact or compete with it. To minimize the influence of endogenous phospholipids in the diet, the type fat that has the least amount of phospholipids was the target. Therefore, PC analysis was performed to quantify the number of phospholipids in different types of fat.

Sample Preparation

The test tubes were labeled, and two samples from each oil (peanut oil, olive oil, corn oil, beef tallow, and lard) were prepared. Approximately, 250 mg of oil was added into a separate test tube, and 2 mL of extraction solvent (chloroform/methanol/water, 1:2:0.8) was added to it. Each sample was homogenized at 30% AMPL for 30 seconds, then, centrifuged at 32,00 x *g* for five minutes. The resultant supernatant was transferred into separate glass tubes. This extraction procedure was repeated twice more, and the resultant supernatant was transferred into the same glass tubes. The combined extract was dried under a Speed-Vap system and re-dissolved in 1 mL of methanol. The solution was filtered using a PTFE membrane (0.45 μm). Finally, the solution was spiked with an internal standard of PC –d9 and Cho-d9 for LC-MS/MS analysis [42].

LC-MS/MS Method

Phospholipids were quantified using Waters (Milford, MA) Acquity H-class separation module. The temperatures of a Waters Acquity UPLC HSS T3 column (2.1 mm x 100mm, 1.7 μm particle size) and the samples were kept at 40 ^{o}C and 10^{o}C respectively. Binary mobile phase system was carried out with Ammonium formate (phase A, 15mM, pH 3.5) and acetonitrile (phase B). The system flow rate was 0.25 mL/min. Linear gradient elution was performed based on the following program: 8% A (initial), 30% A (0-0.1min), 70% A (0.1-7.5min), 95% A (7.5-7.6 min), 95% A (7.6-8.6 min), 8% A (8.6-9 min), and 8% A (9-11 min). The two injection volumes that used for the first and the second sets of samples and standards were 10 μL and 20 μL respectively. The effluent was charged by electrospray ionization (ESI) in a positive mode (+) coupled to tandem mass spectrometry (MS/MS) on a triple quadrupole (TQD) MS. Ionization was performed in a positive mode, with a capillary voltage of 0.6 kV, a cone voltage of 25 V, an extractor voltage of 3 V, and source and desolvation temperatures of 150 ^{o}C and 400^{o}C respectively. Cone and desolvation gases were nitrogen (N2) with flow rates of 20 L/h and 800 L/h respectively. A collision gas for MS/MS was Argon (Ar) used in the collision cell at a flow rate of 0.1 mL/ min. Data acquisition was accomplished using MassLynx software (version 4.1, waters). MS data collection was fixed to 10 points per peak with an average peak width of 6s. The auto-dwell setting was adopted to automatically calculate dwell time based on an interscan delay time of 0.02 s. The Intellistart function of MassLynx was used to develop and optimize multi-reaction monitoring (MRM) parameters for each compound of interest. Compound solutions were directly infused into the ESI+ source (0.1 mg/mL in MeOH/0.1% formic acid at a flow rate of 50 μL/ min) in combination with a background flow of 50% phase A/ 50% phase B at 0.6 mL min. Intellistart automatically selected the most abundant daughter ion, optimized the

source cone voltage, MS/MS collision energy, and generated a single MRM transition for each compound. MRM was performed on parent ions $[M+H]^+$ and signature daughter ions following collision-induced dissociation (CID). MRM mass span was 0.1 Da, the inter-channel delays and inter-scan times were both -1.0 sec. MRM and CID parameters for each compound of interest are listed in table 3.2.1.1 and table 3.2.1.2 All compound peaks were prepared and quantified using the QuanLynx function of MassLynx software.

Table 3.2.1.1 MRM settings for MRM detection of PC derivatives in oil by UPLC-MS/MS

Compound	tR[a] (min)	Molecular Weight (g mol^{-1})	Parent Ion $[M+H]^+$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (Ev)
Acetylcholine	-[b]	145.4	146.4	87.2	25	20
Choline	3.3-4	103.2	104.2	60.1	30	20
Betaine	4-4.5	117.1	118.1	58.2	30	40
Glycerophosphocholine	4-5.2	257	258	104	35	20
Phosphocholine	4-6	183	184	125.1	35	25
Parent[c] 1	2.8-4	-	700-900	184	55	22
Parent 2	2.8-4	-	400-900	184	55	45

[a]: retention time.
[b]: dash (-) indicates "not detected".
[c]: "parent ion sacns" that can ID any PC regardless of mass.

Table 3.2.1.2 MRM settings for MRM detection of PC derivatives in oil by UPLC-MS/MS

Compound	tR[a] (min)	Molecular Weight (g mol^{-1})	Parent Ion $[M+H]^+$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (Ev)
Phosphatidylcholine [16:0/16:1][c]	-	731.8	732.8	184	55	22
Phosphatidylcholine [16:0/16:0]	-	733.9	734.9	184	55	22
Phosphatidylcholine [16:0/18:2]	-	757.9	758.9	184	55	22

Phosphatidylcholine [16:0/18:1]	-	759.9	760.9	184	55	22
Phosphatidylcholine [16:0/20:5, 16:1/20:4]	2.4-2.9	780.1	781.1	184	55	22
Phosphatidylcholine [16:0/20:4]	-	781.9	782.9	184	55	22
Phosphatidylcholine [16:0/20:3]	-	783.9	784.9	184	55	22
Phosphatidylcholine [18:0/18:2, 18:1/18:1]]	-	785.9	786.9	184	55	22
Phosphatidylcholine [18:0/18:1]	-	788	789	184	55	22
Phosphatidylcholine [16:0/22:6, 18:1/22:5, 18:2/20.4]	2.3-3.2	805.9	806.9	184	55	22
Phosphatidylcholine [18:1/20:4, 18:0/20:5, 16:0/22.5]	2.3-3.4	807.9	808.9	184	55	22
Phosphatidylcholine [18:0/20:4]	-	810	811	184	55	22
Phosphatidylcholine [18:0/20:3]	-	810.9	811.9	184	55	22
Phosphatidylcholine [18:1/22:6]	2.4-3	832	833	184	55	22
Phosphatidylcholine [18:0/22:6, 18:1/22:5]	2.4-3	834	835	184	55	22
Phosphatidylcholine [18:0/22:5]	-	835	836	184	55	22
L- Carnitine	4.5-5	161.2	162.2	84.9	34	20
Sphingomyelin (SM) [C16:0]	-	702.7	703.7	184	55	22
Sphingomyelin (SM) [C18:0]	-	730.8	731.8	184	55	22
L-Phosphatidylcholine [C16:0]	-	495.7	496.7	184	55	45
L-Phosphatidylcholine [C18:0]	-	523.8	524.8	1 84	55	45

[a]: retention time.
[b]: dash (-) indicates “not detected”, these species were looked for, but not found in the samples.
[c]: the numbers correspond to the two fatty acids on the PC

3.2.2 Animal Study

Animals

Forty-eight male C57BL/6J mice aged five weeks and weighing approximately 20 g were obtained from Jax (Bar Harbor, ME). The number of mice was determined based on information taken from an article that applied animal studies to estimate TMAO concentrations [12]. For Sample 1 from the article, the average value taken was 125 μmol, and standard deviation taken was 75 μ mol. For Sample 2, 20% reduction was taken to the average (125→100 μ mol) and standard deviation (75→60 μ mol). By adjusting the power equal to 80% and the alpha equal to 5%, the sample size was calculated using Health Care Research Calculators [13]. As a result, it was concluded that eight mice per group are an appropriate sample size for this study. However, the number of mice was increased to twelve (N=12) because three cages per treatment were wished to be used, mistakes during gavage may occur, and to control for multiple comparisons when comparing individual treatments.

These mice were housed for approximately three and half weeks, four mice per cage, in standard vivarium, conditions (12 h light/dark cycle). The day they arrived, they were maintained on standard chow (18% protein, Teklad, Indianapolis, IN) for six days. On the seventh day, they were maintained on the control diet (a low-fat diet) for a week. In the next two weeks, the specific diets (experimental and control diets) were provided and at the end of this period, the meal challenge was applied.

Diets

Sucrose matched diets that are either low or high-fat with or without PC were provided for the 48 mice over 15 days, and a total of 25 kg of chow was used. The mice were divided

equally into four groups, so twelve mice per treatment. Then, they were given free access to food and water. To prevent degradation of fat, diets were maintained at -20°C throughout the experiment, and the food was replaced in feeding cups twice per week. The specific diets were designed as described in **Table 3.2.2.1** to test whether the results of consuming a high-fat with PC diet are significantly different from the results of consuming the other three diets, containing either low-fat, low PC or both low-fat and low PC. In this case, Diet 2, Diet 3 and Diet 4 are the controls. This experimental design is termed a 2 x 2 factorial because there are two levels in the first factor and two levels in the second factor

Table 3.2.2.1 Sucrose match diets

	High-Fat (F) 60%	**Low-Fat (F) 10%**
High PC	High F - High PC diet (1)	Low F- High PC diet (2)
Low PC	High F – Low PC diet (3)	Low F – Low PC diet (4)

Animals were provided with either a low-fat diet (10% kcal from fat) or high-fat diet (60% kcal from fat). Both diets were from the diet-induced obesity (DIO) model series (Research Diets, Inc., New Brunswick, NJ). Both diets contained equal amounts of sucrose. Diets typically contain both lard and soybean oil. To eliminate endogenous phospholipids, diets were modified to contain corn oil and lard only without soybean oil content, as soybean oil contains significant levels of PC. Within these diets, mice received the diets either without or with soybean L-α-Lecithin, consisting of more than 97.7% PC and 0.3% triglycerides [67], (0 or 0.5% by weight of the diet). The following **Table 3.2.2.2** has important information about the two diets: a low-fat diet (LF, Research Diets D15051801), a high-fat diet (HF, Research Diets D07012601).

Table 3.2.2.2 Research Diets, Four DIO (Diet-Induced Obesity) Diets with Fat Modifications with/without 0.5 gm% Granular Soybean Lecithin.

Product #	**HF** D07012601 Red		**LF** D15051801 Orange	
	gm %	**Kcal%**	**gm %**	**Kcal%**
Protein	26	20	19	20
Carbohydrate	26	20	67	70
Fat	35	60	4	10
Total		100		100
Kcal/gm			3.8	
Ingredient	**gm**	**Kcal**	**gm**	**Kcal**
Casein, 30 Mesh	200	800	-	-
Casein, 80 Mesh	-	-	200	800
L-Cystine	3	12	3	12
Corn Starch	0	0	506.2	2025
Maltodextrin 10	125	500	125	500
Sucrose	68.8	275	68.8	275
Cellulose, BW200	50	0	50	0
Soybean oil	0	0	0	0
Corn oil	25	225	25	225
Lard	245	2205	20	180
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0	0	0.025	0
FD&C Red Dye #40	0.05	0	0.025	0
Total	773.85	4057	1055.1	4057

Half of the low-fat diet was prepared with soybean L-α-Lecithin (0.5% by weight of the diet), and green color was added to the new diet to make it different from the low-fat diet that didn't contain Lecithin. Similarly, half of high-fat diet was prepared with soybean L-α-Lecithin (0.5% by weight of the diet), and a violet color was added to the diet to make the new diet different from the high-fat diet that didn't contain Lecithin.

Meal Challenges

A high-fat high-PC meal challenge was provided to the four groups to indirectly observe how the mice's gut microbiota adapted to the altered diet. The meal contained 9.99 g of corn oil and 0.75 g soybean L-α-Lecithin per 1 kg body weight. The meal was administered by intragastric gavage (0.1 mL/ 10 g) using a curved stainless steel gavage needle (20 Gauge, 3.8 cm length, 2.4 mm ball tip) attached to a 3 mL syringe [11]. Ice with alcohol was used to numb the affected area before tail snips with sharp surgical scissors. The blood collections were over 12 hours: before the meal challenge, every hour for the first four hours after the meal challenge, and every 2 hours after that. Mice remained on water alone until the last blood sample was collected. Then, food was restored (see **Figure 3.2.2.1).**

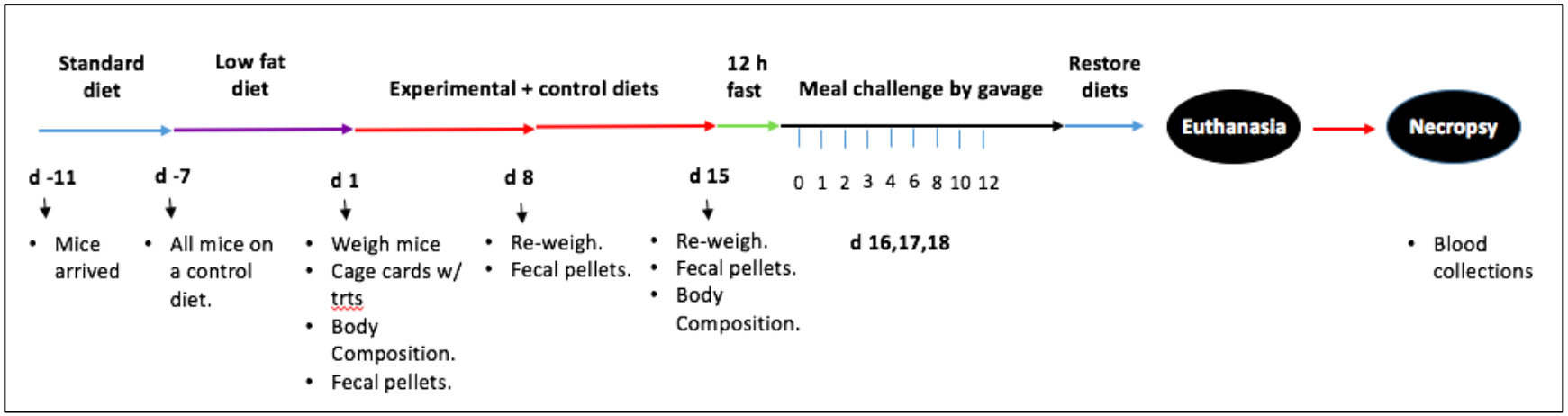

Figure 3.2.2.1 Illustration of how the animal study was performed.

3.2.3 Samples and Measurements

Body mass (BW)

Mouse BW was measured three times throughout the study, day 1, day 8, and day 15. The mice were individually placed inside an empty cage lid on a tared lab balance.

Body composition

Mouse MRI body composition was measured during day 1 and day 15 using a Bruker LF90 NMR analyzer as described in the manufacturer's instructions. This analysis measured lean tissue, fat, and free fluid mass of a mouse in grams and % at different stages of manipulating the diets. Mice were individually placed in the restraining chamber for approximately 1 to 2 minutes while the analyzer took measurements.

Samples

- **Blood collection before and after the acute meal:**

The total number of blood collections was nine. Blood drops were collected inside microcentrifuge tubes before the meal challenge and at the first, second, third, and fourth hours, and every two hours over 12 hours. The amount of blood collected was 20 uL each time. Therefore, the total blood collected was 20*9= 180 uL (180 uL = 0.18 mL), which is ~2.765 mL for a 35 g mouse. Blood tubes were stored at −80°C.

- **Fecal pellets:**

Fecal pellets were collected three times (two tubes per a cage) during day 1, day 8, and day 15. Tubes were stored at −80°C.

Euthanasia and non-fasting blood collection

At the end of this study, mice were maintained on their different diets for few days to return weights. Then, they were euthanized individually by CO_2 (following AVMA Guidelines on Euthanasia). Blood was immediately collected by cardiac puncture, (using 1mL Luer-Lok™ syringes and 0.6mm x 25mm gauge needles). Then, blood was left to clot at room temperature for 30 mins inside BD Vacutainer® serum separation tubes with Clot Activator. Clotted blood was centrifuged (10 min, 1,500 x *g*), and the resulting serum was stored at −80°C.

3.2.4 Sample Preparation and Analysis

Fecal pellets extraction of TMA and its' derivations

First, a fecal pellet was weighed inside a centrifuge tube after taring the weight of the tube, and the pellet weight was recorded. Then, 300 μL of methanol (MeOH) was added into the tube. The fecal pellet was crushed using lab spatula. It was sonicated for 5 min. It was centrifuged at 17,000 x *g* for 3 min. The supernatant was collected. This procedure was repeated by adding another 300 μL of MeOH, sonicating for 5 min, vortexing for few seconds, and centrifuging at 17,000 x *g* for 3 min. Finally, the supernatant was collected into the same tube.

TMAO, Betaine, Choline, and L-carnitine Analysis Protocol

- **Internal standard (IS) solution preparation:**

IS solution was made of 25 μM Choline-d9, 25 μM Betaine-d9, 25 μM TMAO-d9, and 120 μM L-carnitine-d9 combined.

- **Sample Preparation for non-fasting blood:**

First, plasma samples were thawed at room temperature. Microcentrifuge tubes were prepared with 25µL plasma and 300µL of acetonitrile (ACN)/IS solution combined. Samples were vortexed for approximately 1 min at level 8. Then, they were centrifuged at 17,000 x *g* for 3 minutes at room temperature using benchtop micro-centrifuge. An amount of 200µL was transferred from microcentrifuge tube to UPLC vials with inserts without pulling up any precipitate using a 0.22 µm Nylon filter. Vials were analyzed on LC-MS (see LC-MS/MS Method). Plasma samples were refrozen at -80°C.

- **Sample Preparation for fecal pellets:**

First, tubes with fecal pellet extractions were thawed at room temperature. Microcentrifuge tubes were prepared with 25µL extraction and 300µL of ACN/IS solution combined. Tubes were vortexed for approximately 1 min at level 8. Then, they were centrifuged at 17,000 x *g* for 3 minutes at room temperature using benchtop micro-centrifuge. An amount of 200µL was then transferred from microcentrifuge tube to UPLC vials with inserts without pulling up any precipitate using a 0.22 µm Nylon filter. Vials were analyzed on LC-MS (see LC-MS/ MS Method). Fecal pellet extractions were refrozen at -80°C.

- **Sample Preparation for blood time points:**

First, blood samples were thawed at room temperature. Microcentrifuge tubes were prepared with 10µL blood and 100µL of ACN/IS solution combined. Samples were vortexed for approximately 1 min at level 8. They were sonicated for 5 min. Then, they were centrifuged at 17,000 x *g* for 3 minutes at room temperature using benchtop micro-centrifuge. An amount of

200µL was then transferred from microcentrifuge tube to UPLC vials with inserts without pulling up any precipitate using a 0.22 µm Nylon filter. Vials were analyzed on LC-MS (see LC-MS/ MS Method). Vials were frozen at -80°C.

TMA analysis protocol

- **Sample Preparation:**

First, non-fasting blood/ fecal pellet extractions samples were thawed at room temperature. Microcentrifuge tubes were prepared with 20µL samples, 20µL IS solution (25 µM TMA-d9 HCl in water), 8µL concentrated ammonia, and 120µL ethyl bromoacetate solution (20 mg/mL in ACN). Then, they were left to incubate at room temperature for 30 min. An amount of 800µL of ACN/formic acid (aqueous 50% ACN/0.025% formic acid) was added to the tubes after incubation. They were centrifuged at 17,000 x *g* for 5 minutes at room temperature using benchtop micro-centrifuge. Approximately, 800µL were transferred from microcentrifuge tube to UPLC vials without pulling up any precipitate using a PTFE 4 mm, 0.2 µm filter. Vials were analyzed on LC-MS (see LC-MS/ MS Method). Vials were frozen at -80°C.

LC- MS/MS solvents and conditions

UPLC solvents and conditions are Mobile Phase A: 15mM mmonium formate (pH adjusted to 3.5), Mobile Phase B: acetonitrile with flow rate: 0.65 mL/min (percentage: 20% (A), 80% (B)), gradient: isocratic, column temperature: 30°C (column: waters BEH HILIC 2.1x100mm, 1.7µm) and (guard column: waters BEH HILIC 2.1x5mm, 1.7µm), sample temperature: 10°C, running time: 3 minutes, and Inject Volume: 25µL.

LC-MS/MS Method

TMAO and its precursors were quantified by stable isotope dilution LC-MS using Waters (Milford, MA) Acquity H-class separation module. Deuterium internal standard (d9) was added into samples at known amounts to give a measure of control for extraction without interfering with the actual compounds in the samples. The temperature of a Waters Acquity UPLC HSS T3 column (2.1 mm x 100mm, 1.7 μm particle size) and the samples were kept at 30^{o}C and 10^{o}C respectively. Binary mobile phase system was carried out with Ammonium formate (phase A) and acetonitrile (phase B). The system flow rate was 0.5 mL/min. Linear gradient elution was performed based on the following program:20% A (initial) and 20% A (3 min). The effluent was charged by electrospray ionization (ESI) in a positive mode (+) coupled to tandem mass spectrometry (MS/MS) on a triple quadrupole (TQD) MS. TMA was derivatized with ethyl bromoacetate to form betaine bromide before ionization to increase the ionization efficiency and to improve the reproducibility of TMA fragmentation because it is not naturally charged [79]. Ionization was performed in a positive mode, with a capillary voltage of 0.6 kV, a cone voltage of 25 V, an extractor voltage of 3 V, and source and desolvation temperatures of 150 ^{o}C and 400^{o}C respectively. Cone and desolvation gasses were N2 with flow rates of 20 L/h and 800 L/h respectively. A collision gas for MS/MS was Ar used in the collision cell at a flow rate of 0.1 mL/ min. Data acquisition was accomplished using MassLynx software (version 4.1, waters). MS data collection was fixed to 10 points per peak with an average peak width of 6s. Dwell time was automatically calculated using the auto-dwell setting based on an inter-scan delay time of 0.02 s. The Intellistart function of MassLynx was used to develop and optimize multi-reaction monitoring (MRM) parameters for each compound of interest. Compound solutions were directly infused into the ESI+ source (0.1 mg/mL in MeOH/0.1% formic acid at a flow rate of 50 μL/

min) in combination with a background flow of 50% phase A/ 50% phase B at 0.6 mL min. Intellistart automatically selected the most abundant daughter ion, optimized the source cone voltage, MS/MS collision energy, and generated a single MRM transition for each compound. MRM was performed on parent ions $[M+H]^+$ and signature daughter ions following collision-induced dissociation (CID). MRM mass span was 0.2 Da, the inter-channel delays and inter-scan times were both -1.0 sec. MRM and CID parameters for each compound of interest are listed in tables 3.2.4.1 and 3.2.4.2. All compound peaks were prepared and quantified using the QuanLynx function of MassLynx software.

Table 3.2.4.1. MRM settings for MRM detection of TMA in non-fasting blood and fecal pellets by UPLC-MS/MS

Compound	tR[a] (min)	Molecular Weight (g mol^{-1})	Parent Ion $[M+H]^+$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
TMA	0.5-1	145.27	146.27	118.12	34	16
TMA-D9	0.5-1	154.33	155.33	127.21	34	20

[a]: retention time

Table 3.2.4.2. MRM settings for MRM detection of TMAO and its derivations in non-fasting blood and fecal pellets by UPLC-MS/MS

Compound	tR[a] (min)	Molecular Weight (g mol^{-1})	Parent Ion $[M+H]^+$ (m/z)	Daughter Ion (m/z)	Cone Voltage (V)	Collision Energy (eV)
TMAO	0.9-1.2	75.16	76.16	58.91	40	10
TMAO-D9	0.9-1.2	84.22	85.22	68.1	40	12
L-carnitine	1.1-1.6	161.26	162.26	84.99	34	20
L-carnitine-D9	1.1-1.6	170.28	171.28	84.99	34	20

Choline	0.7-0.9	103.2	104.2	60.02	38	16
Choline-D9	0.7-0.9	112.32	113.32	69.08	40	16
Gamma-butyrobetaine	1.1-1.6	145.27	146.27	87	26	16
Betaine	0.8-1.1	117.24	118.24	59.42	44	18
Betaine-D9	0.8-1.1	126.3	127.3	68.1	46	18

[a]: retention time.

Standard Curve Preparation Instructions for TMAO and TMA Analysis

- **TMAO standard curve preparation:**

The stock solution was made of TMAO (18.8mg), betaine (29.3mg), choline chloride (34.9mg), L-carnitine HCL (49.4mg), γ-butyrobetaine HCl (45.4mg), and MQ water. The five standards were added into a 500 mL volumetric flask, and the actual masses weighed were recorded (within ± 1 mg of the specified value). The volumetric flask was filled with MQ water into volume 500 mL. The flask was stirred and sonicated to dissolve the masses added. Fifteen microcentrifuge tubes*2 and 15 HPLC vials were labeled from #1 to #15. Then, one mL of MQ water was added into tubes from # 2 to #15, but not tube #1. One mL of stock solution was placed in tube #1. Half mL of the stock solution from tube #1 was placed into tube #2. Another half mL of tube #2 was placed into tube#3. Serial dilution was continued by the same procedure until tube #15 was completed. All fifteen tubes were capped and vortexed. Then, they were analyzed using TMAO method (see TMAO analysis protocol). Tubes from #1 to # 15 were frozen at -80°C.

- **TMA standard curve preparation**

The stock solution was made of TMA(17.0mg) and MQ water. TMA standard was added into a 500 mL volumetric flask, and the actual mass weighed was recorded (within ± 1 mg of the specified value). The volumetric flask was filled with MQ water into volume 500 mL. The flask was stirred and sonicated to dissolve the mass added. 15 microcentrifuge tubes*2 and 15 HPLC vials were labeled from #1 to #15. Then, one mL of MQ water was added into tubes from # 2 to #15, but not tube #1. One mL of stock solution was placed in tube #1. Half mL of the stock solution from tube #1 was placed into tube #2. Another half mL of tube #2 was placed into tube#3. Serial dilution was continued by the same procedure until tube #15 was completed. All fifteen tubes were capped and vortexed. Then, they were analyzed using TMA method (see TMA analysis protocol). Tubes from #1 to # 15 were frozen at -80°C.

TMA, TMAO, Betaine, gamma-Butyrobetaine, Choline, and L-carnitine Deuterium internal standard chromatograph.

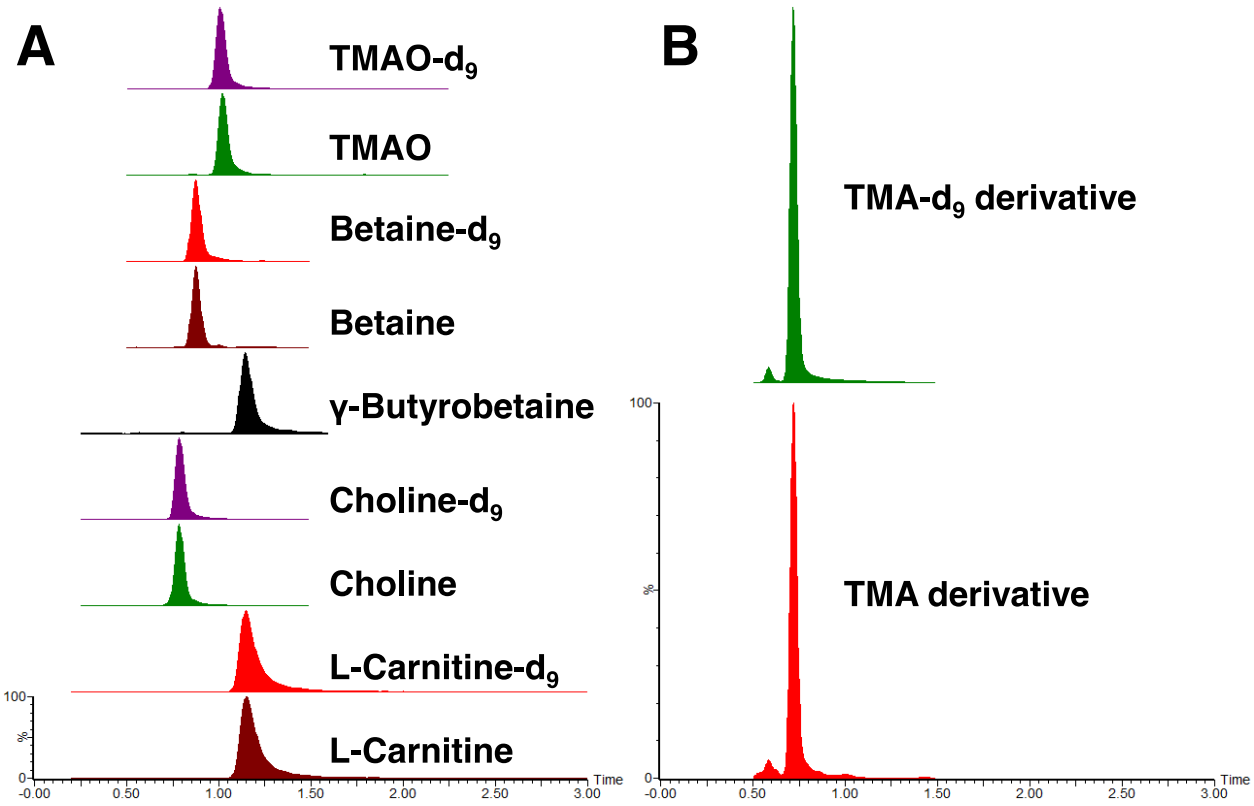

Figure 3.2.4.1 Deuterium internal standard chromatograph for TMA, TMAO, betaine, gamma-butyrobetaine, choline, and L-carnitine used as an isotope to give a measure of control for extraction. TMA-d_9 is the internal standard for TMA. TMAO-d_9 is the internal standard for TMAO. Cholin-d_9 is the internal standard for choline. L-carnitine-d_9 is the internal standard for L-carnitine. Betaine-d_9 is the internal standard for betaine and gamma-butyrobetaine.

3.2.5 Statistical Analysis

Two-way ANOVA test compares the mean differences between groups when two independent variables are present [69]. In this study, it was used to analyze the main effects of the two factors fat and PC and the interaction between the two factors. Tukey's Post hoc test was used to compare their means. Alpha that refers to significance level, the probability of making type 1 error, was equal to 0.05 all the time. Statistical analysis was performed on Prism v6 (GraphPad, La Jolla, CA). Outliers were identified using Dixon Q test.

3.3 Results and Discussion

3.3.1 Quantification of Phospholipids

Looking at figures 3.3.1.1, 3.3.1.2, 3.3.1.3, 3.3.1.4, 3.3.1.5, 3.3.1.6, 3.3.1.7, 3.3.1.8, 3.3.1.9, 3.3.1.10, 3.3.1.11, 3.3.1.12, and 3.3.13 peaks areas roughly correspond to the concentrations of phospholipids in the samples. While we did not quantify the phospholipids, these data were used qualitatively and semi-quantitatively to identify dietary lipids with the lowest endogenous phospholipid concentrations. In **Figure 3.3.1.1,** the only one that had a significant peak for phosphocholine was the egg. Lard had a small peak only. The chromatograms for the rest of the samples showed some noise, which indicates for not present amounts. However, in **Figure 3.3.1.2,** all samples showed significant peaks for glycerophosphocholine, except corn oil didn't show any peak. Olive oil had a small peak only. Similarly, all samples in **Figure 3.3.1.4** had significant peaks for choline except corn oil. As well as the last figure, olive oil result showed a small peak. In **Figure 3.3.1.3,** all samples showed small peaks for betaine except corn oil and beef tallow didn't show any peak. There were no detectable levels of acetylcholine shown **in Figure 3.3.1.5**. For parent 1 and parent 2 in **Figure**

3.3.1.6 and **Figure 3.3.1.7**, most samples showed significant peaks. However, corn oil and olive oil didn't show any detectable peaks. In **Figure 3.3.1.8** and **Figure 3.3.1.11,** the amount of phosphatidylcholine was significantly high in egg and small in lard. However, the rest of the samples didn't show any detectable levels of phosphatidylcholine. Significant levels of phosphatidylcholine were detected in all samples in **Figure 3.3.1.9** and **Figure 3.3.1.10.** Looking at **Figure 3.3.1.12,** small levels were observed in all samples, but a significant peak was shown with egg. In **Figure 3.3.1.13,** small levels of L-carnitine were observed with beef tallow, peanut oil, corn oil, and olive oil. Otherwise, egg and lard results showed significant peaks. The results shown in figures showed that corn oil and lard compared to the others have the least amount of phospholipids. Therefore, they were used in both of the chronic diets (high-fat and low-fat diets), replacing the soybean oil typically used in these diet formulations, to minimize the influence of endogenous phospholipids, as we wished to add PC separately to control the levels of phospholipids, see **Table 3.2.2.2**.

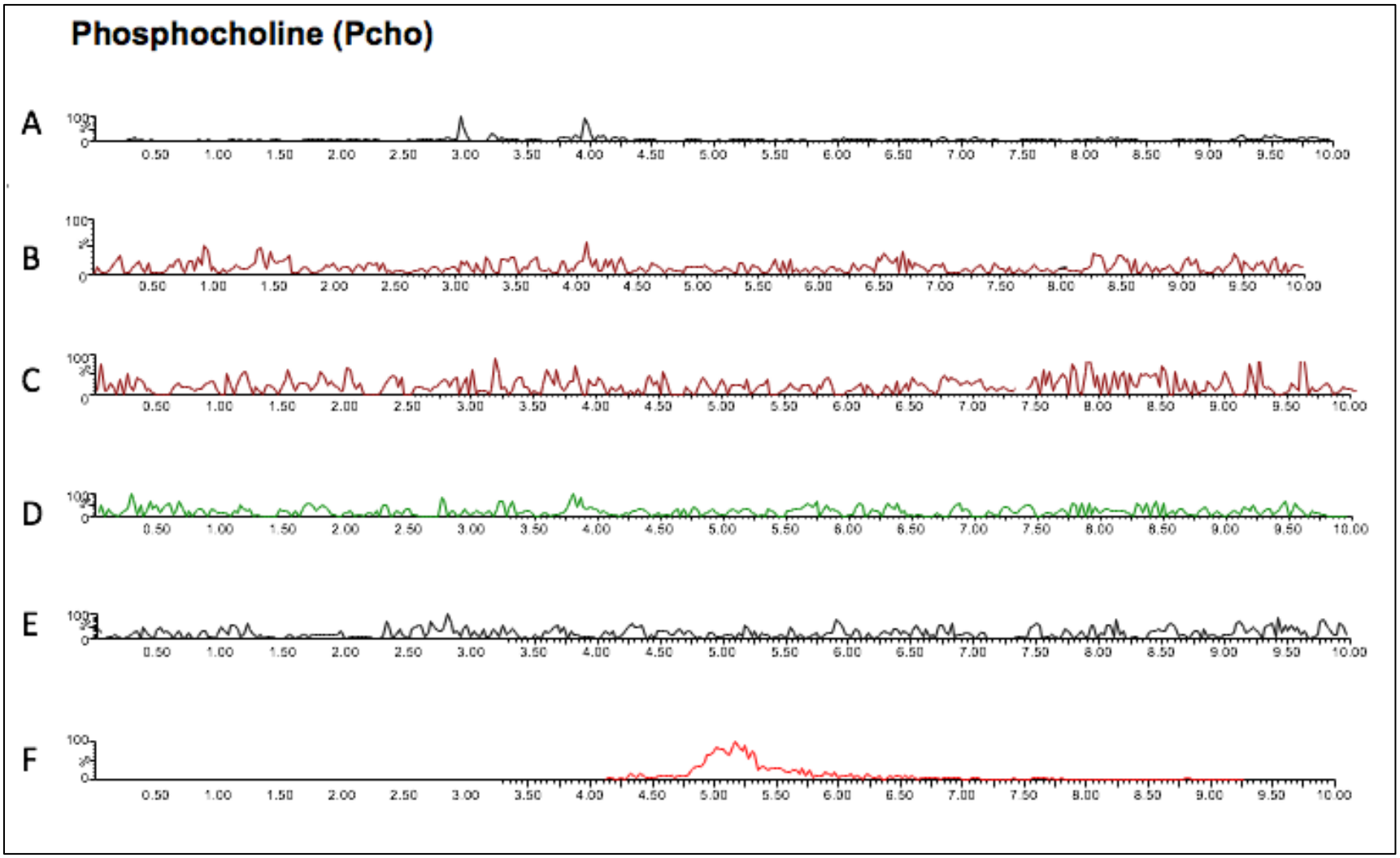

Figure 3.3.1.1 Phosphocholine chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

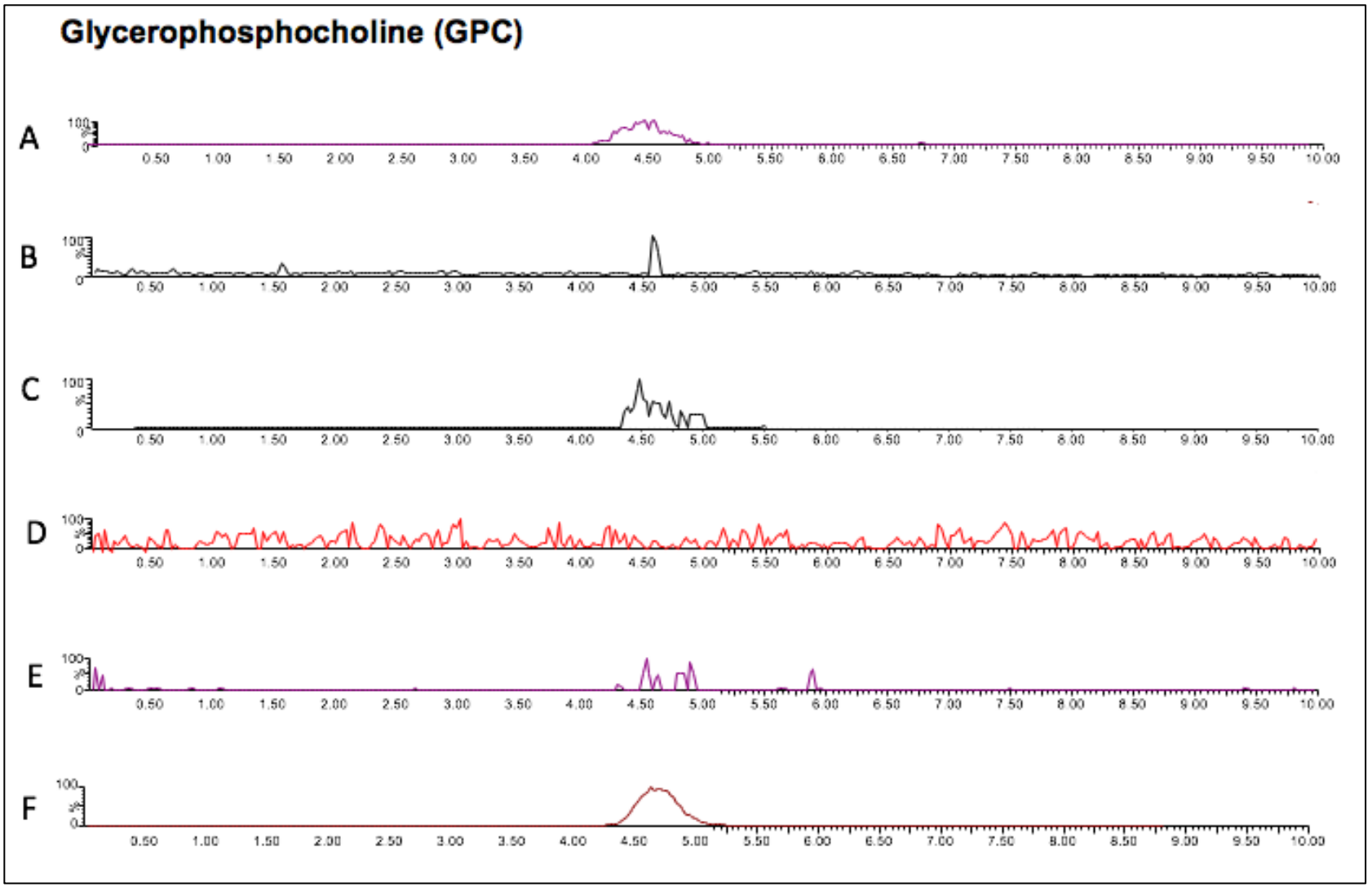

Figure 3.3.1.2 Glycerophosphocholine chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

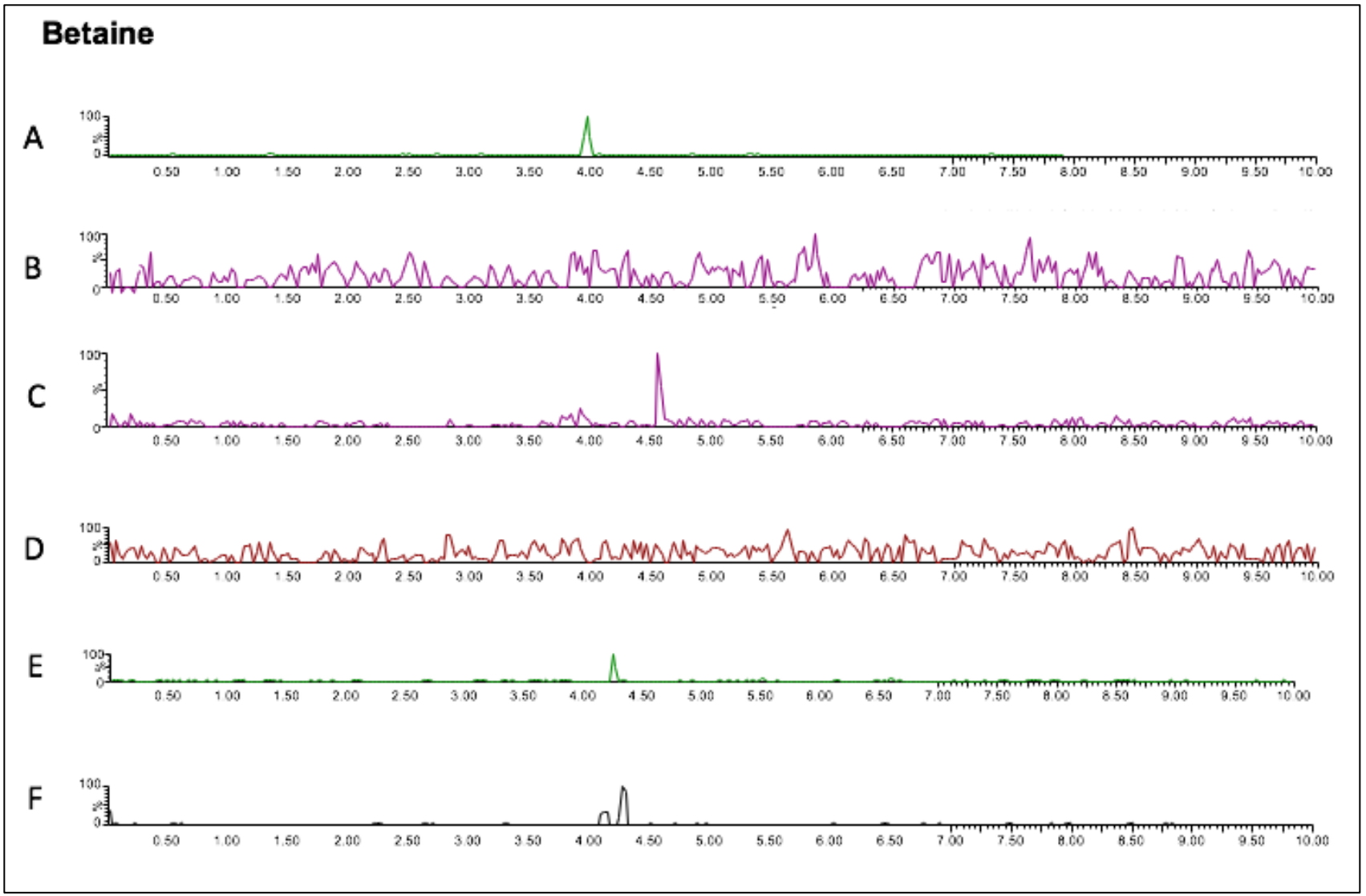

Figure 3.3.1.3 Betaine chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

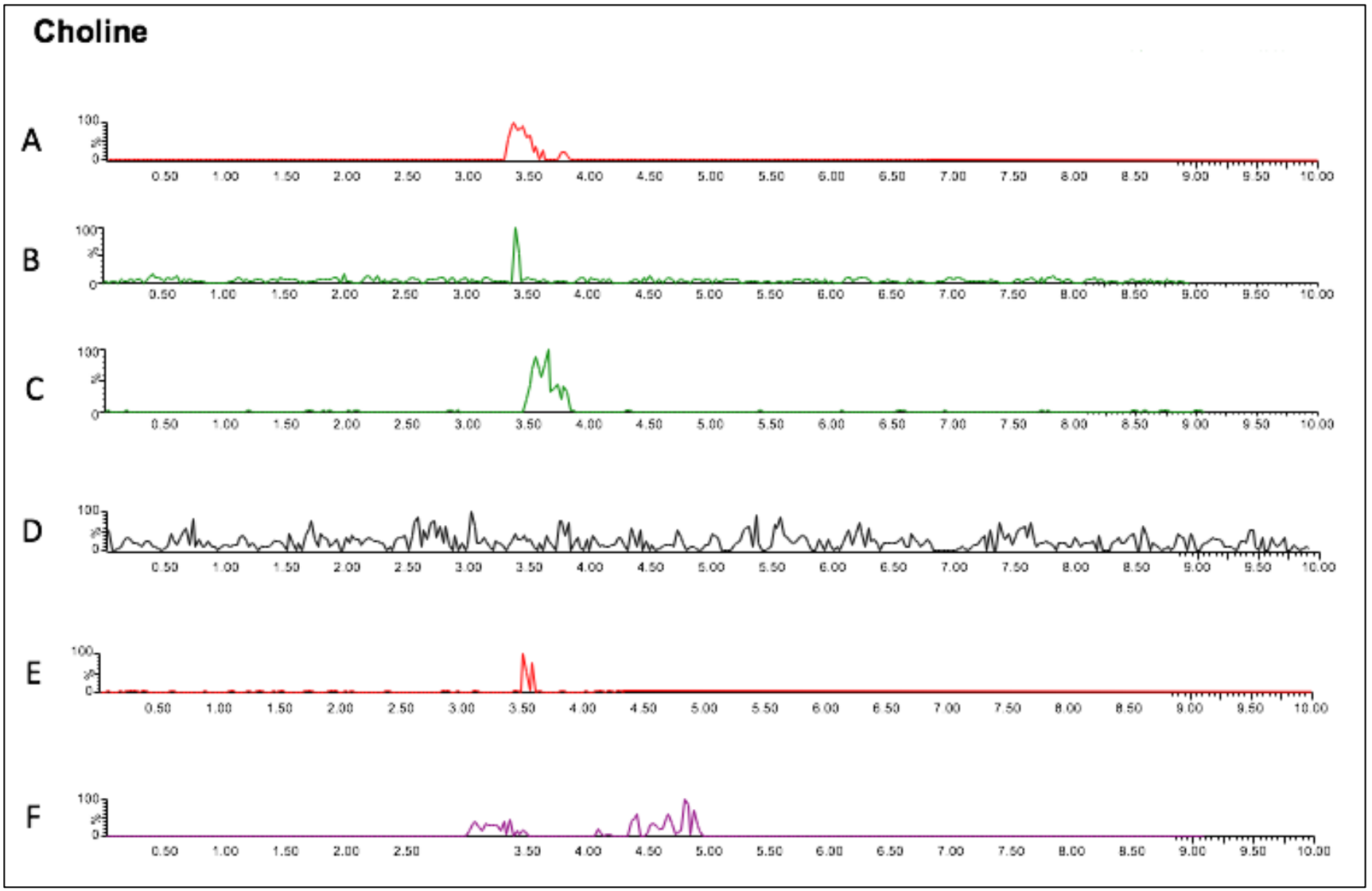

Figure 3.3.1.4 Choline chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

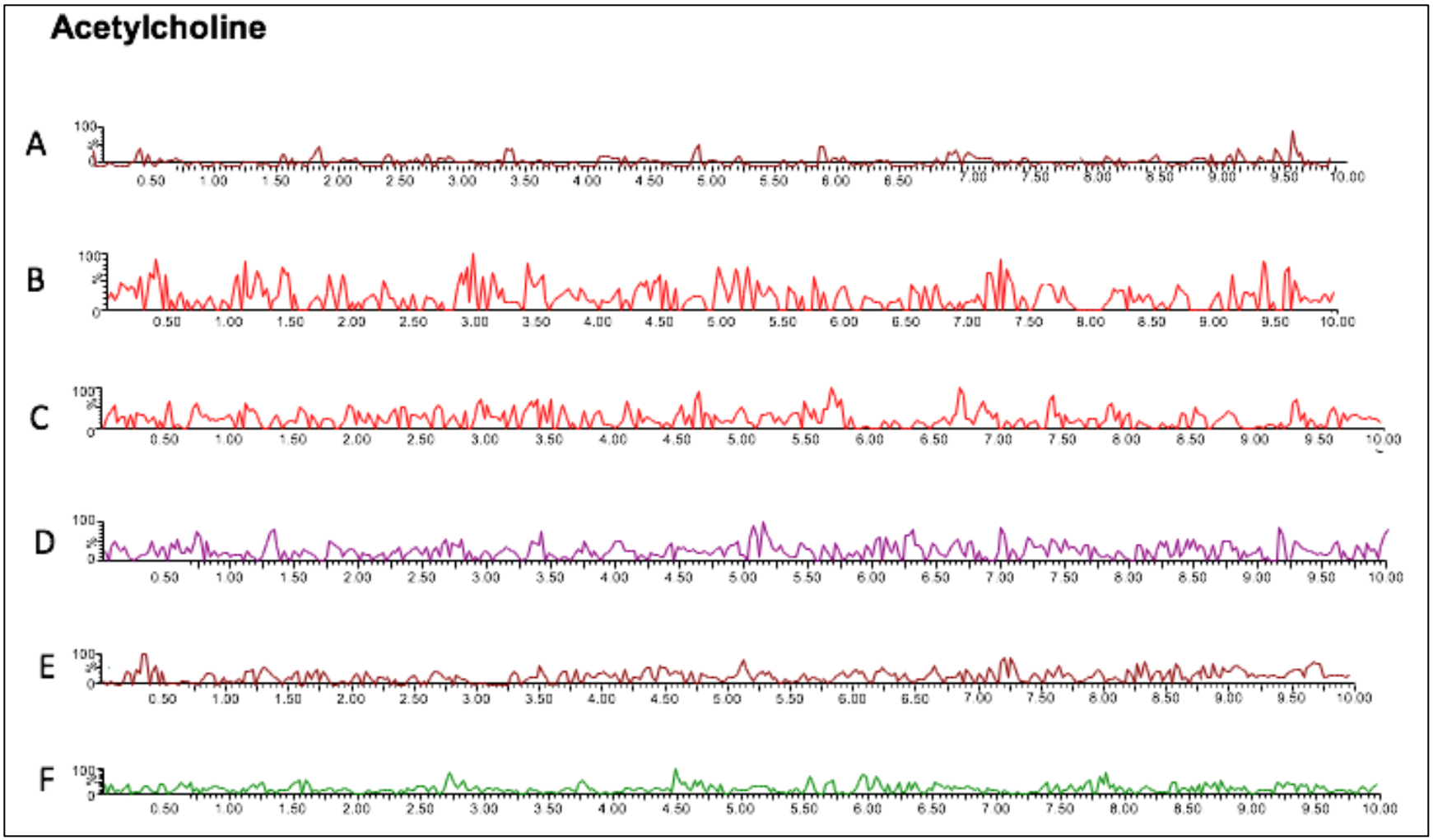

Figure 3.3.1.5 Acetylcholine chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

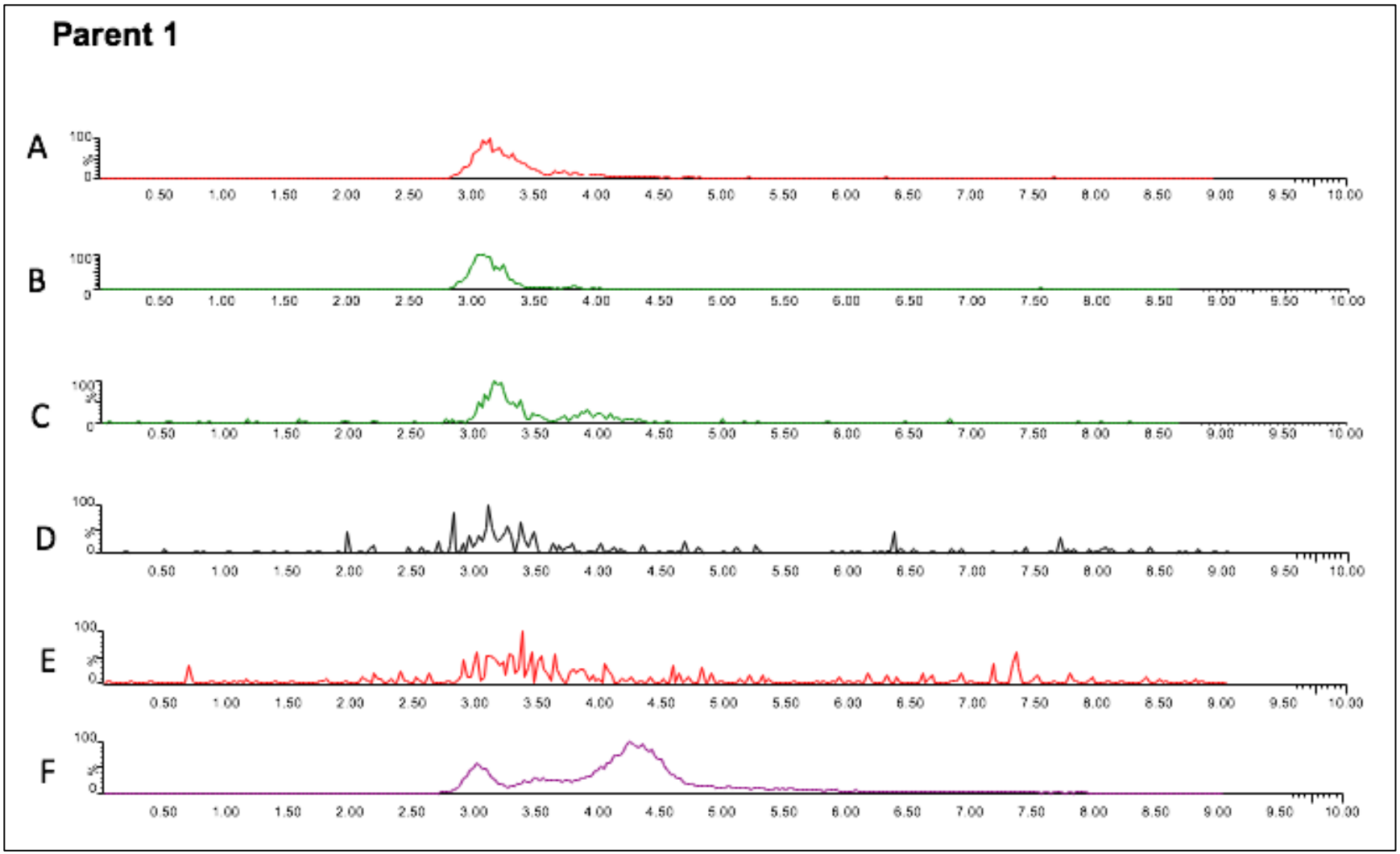

Figure 3.3.1.6 Parents1 chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

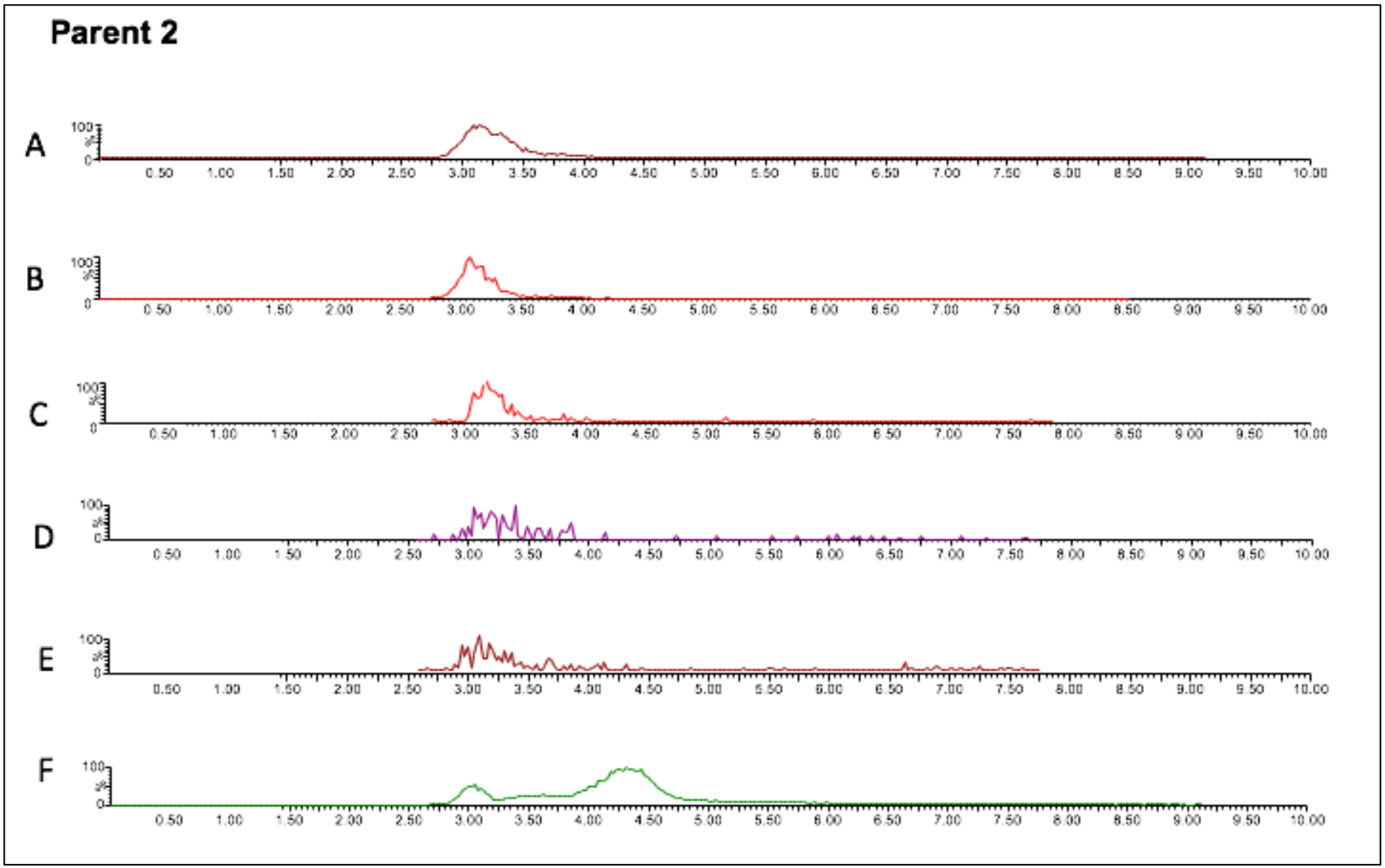

Figure 3.3.1.7 Parents1 chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

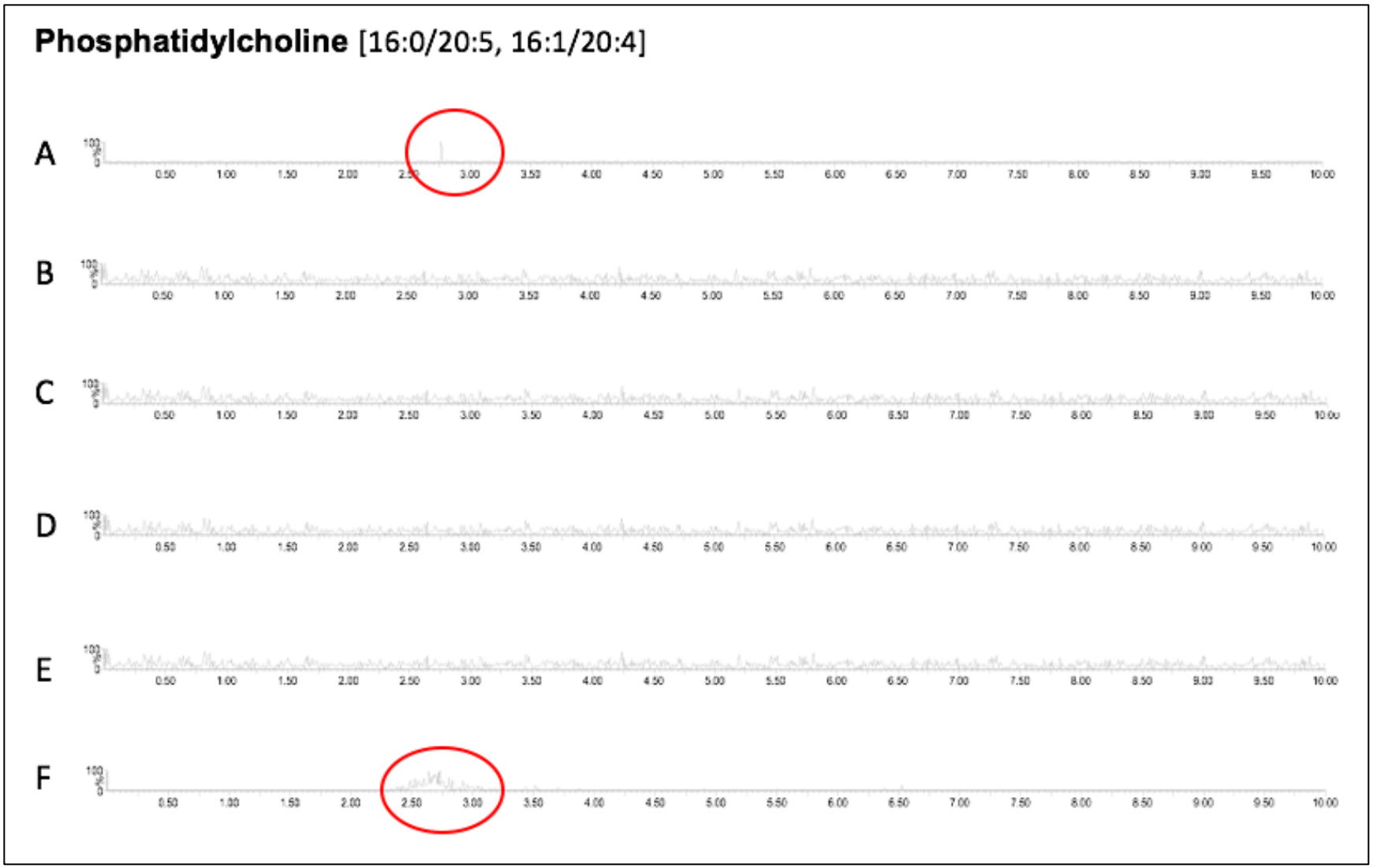

Figure 3.3.1.8 Phosphatidylcholine [16:0/20:5, 16:1/20:4] chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

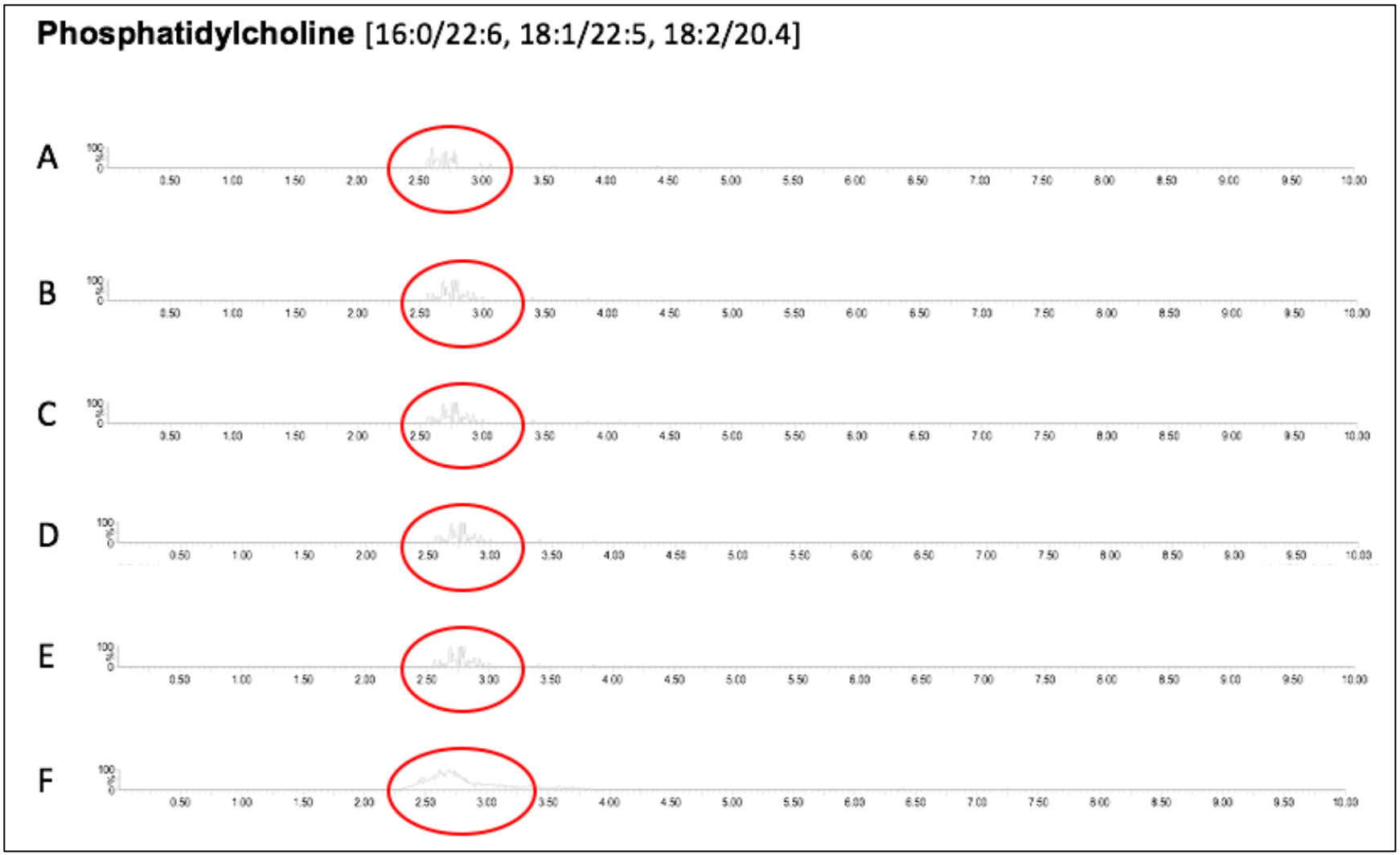

Figure 3.3.1.9 Phosphatidylcholine [16:0/22:6, 18:1/22:5, 18:2/20.4] chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

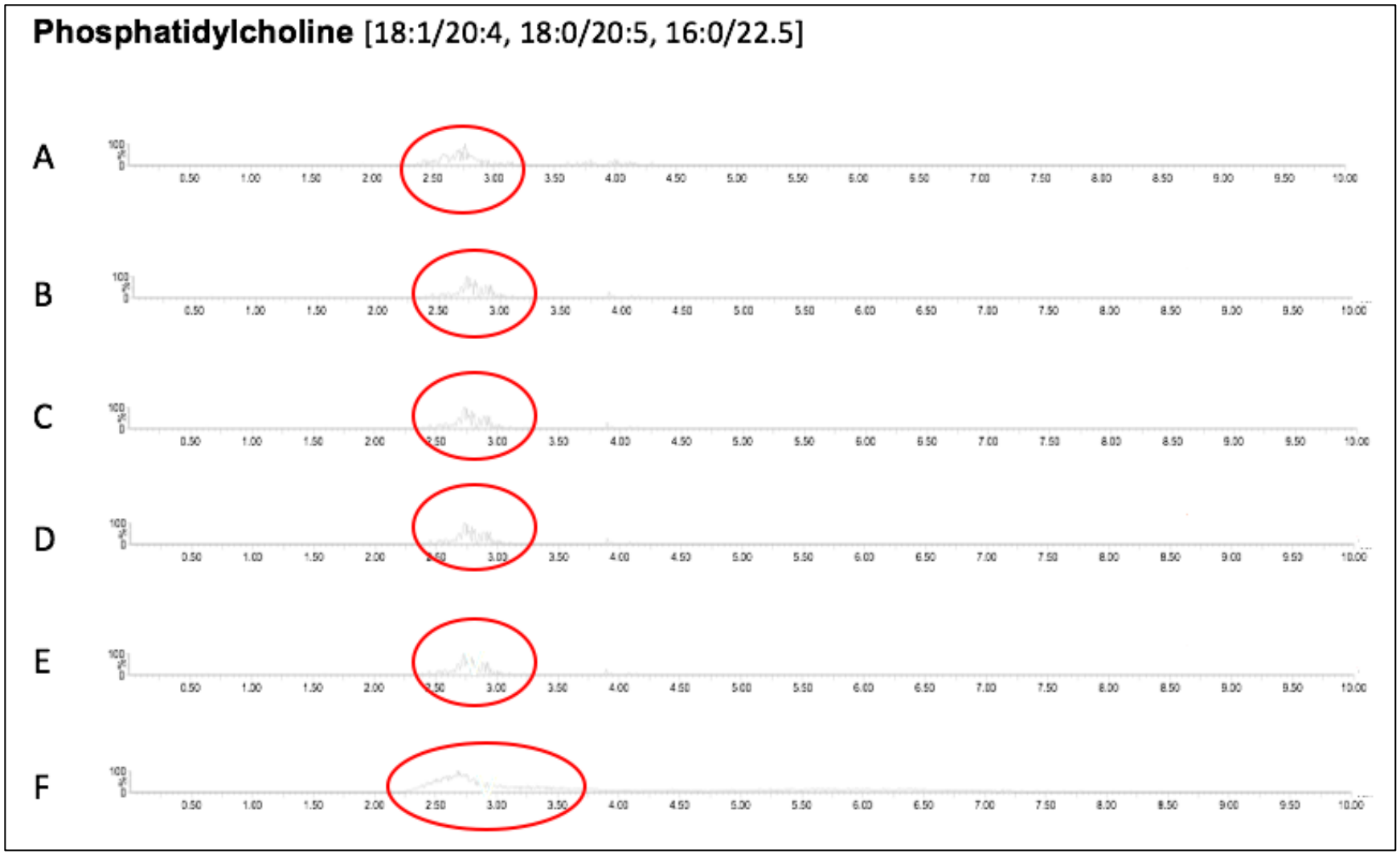

Figure 3.3.1.10 Phosphatidylcholine [18:1/20:4, 18:0/20:5, 16:0/22.5] chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

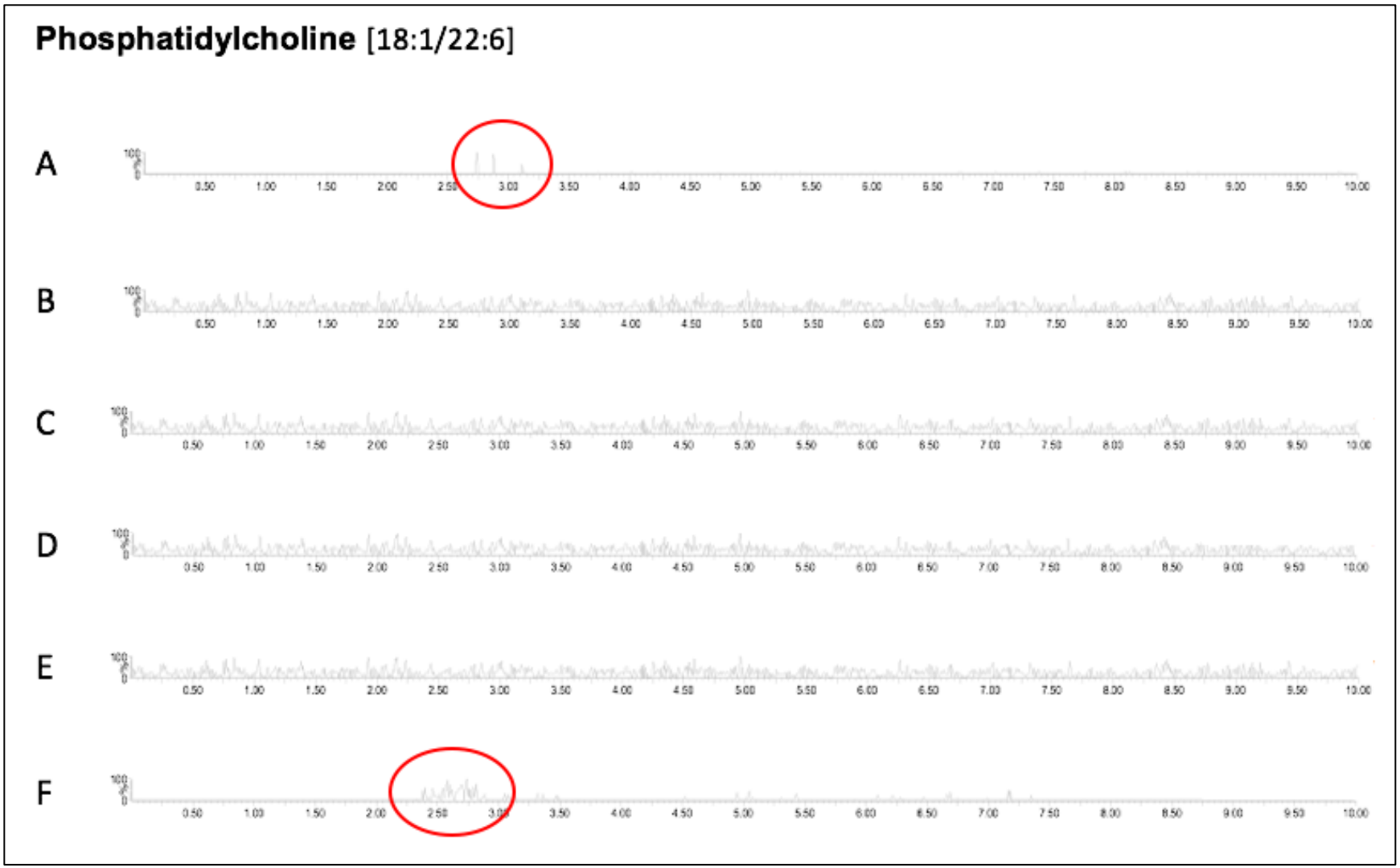

Figure 3.3.1.11 Phosphatidylcholine [18:1/22:6] chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

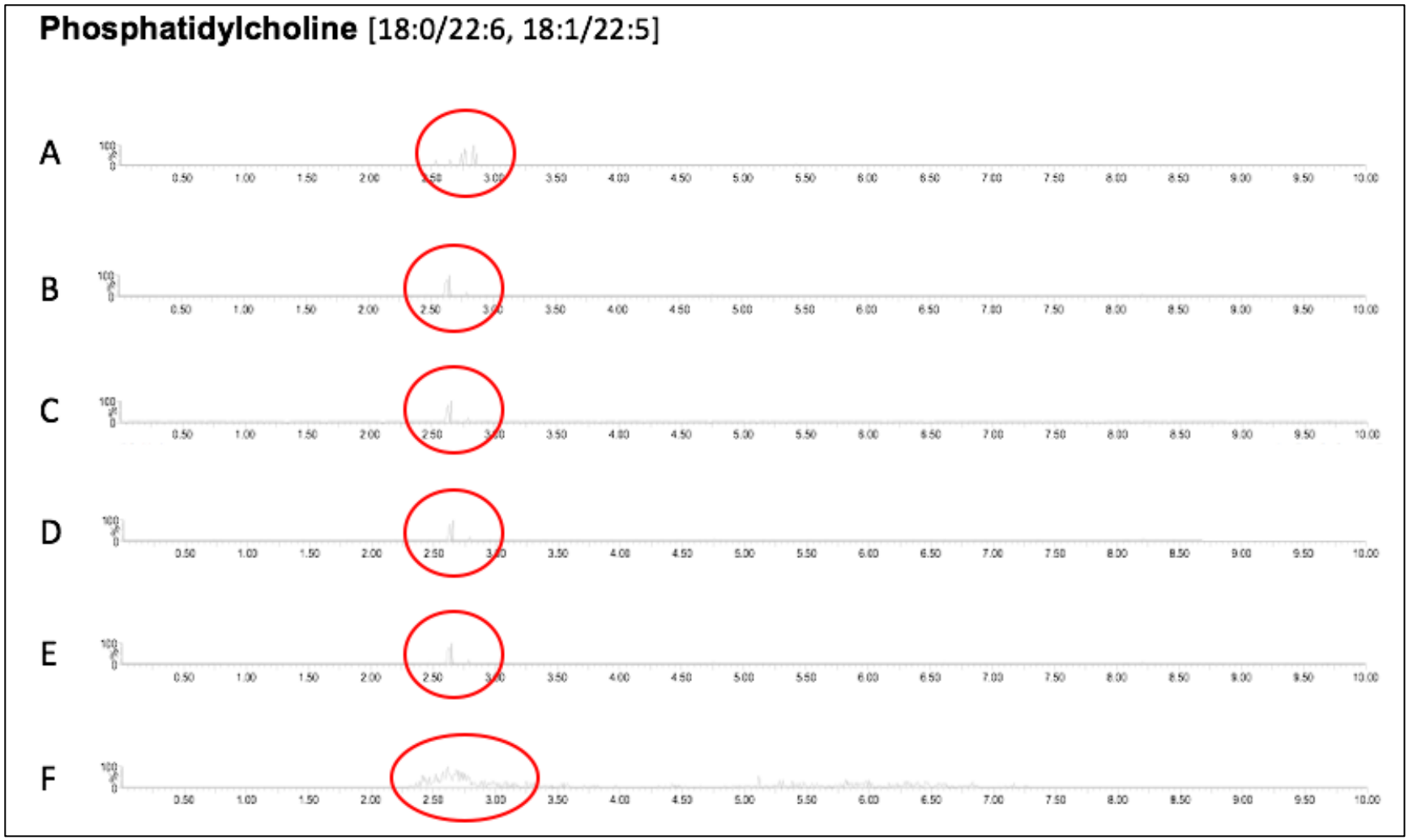

Figure 3.3.1.12 Phosphatidylcholine [18:0/22:6, 18:1/22:5] chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Egg.

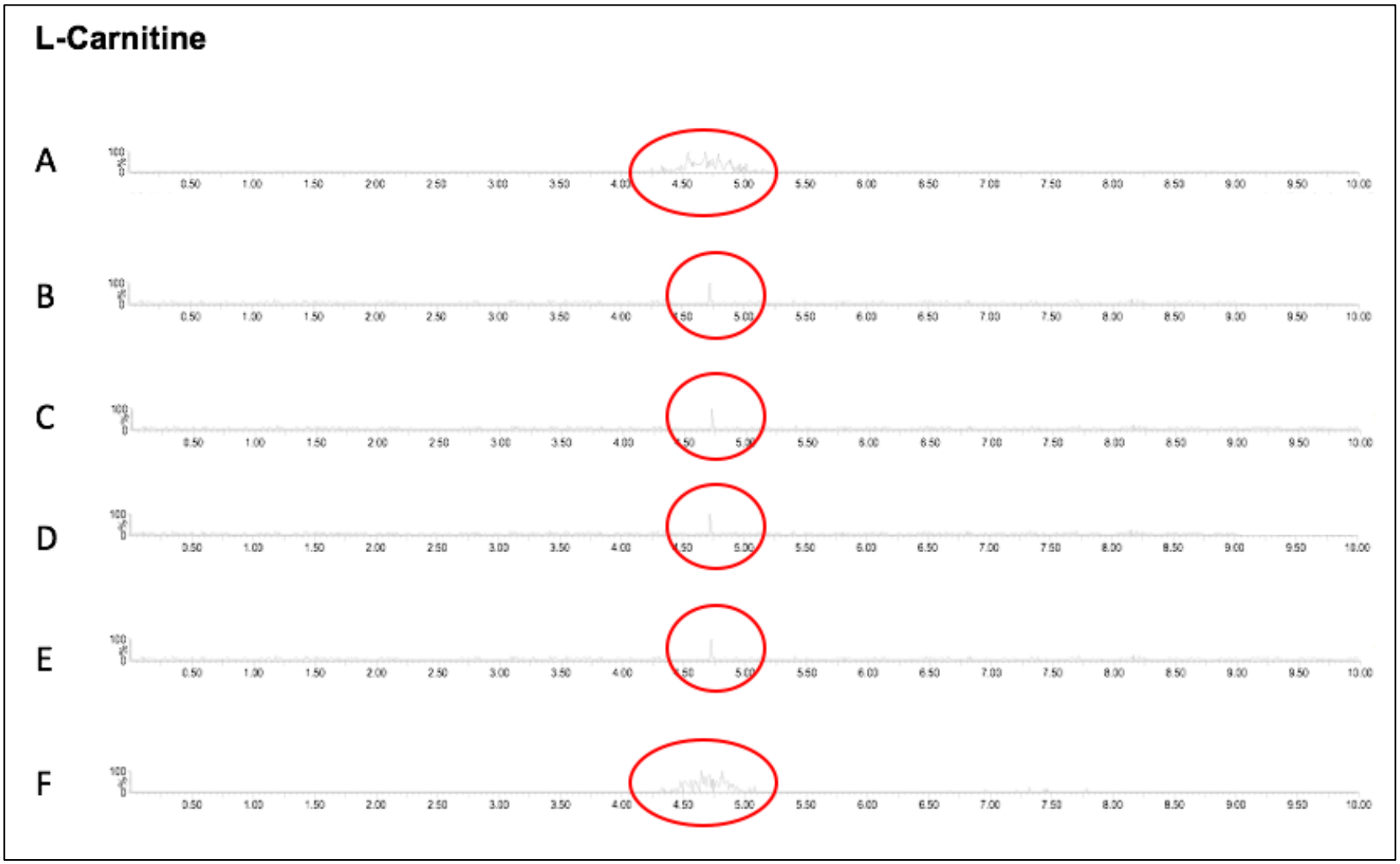

Figure 3.3.1.13 L-carnitine chromatogram: A - lard, B - Beef Tallow, C - Peanut oil, D - Corn oil, E - Olive oil, F - Eg

3.3.2 Food intake.

The average food intake by a mouse in each treatment was displayed in **Table 3.3.2.** The illustrated data shows that the intake by mice in both of the low-fat treatments was higher than the intake by mice in both of the high-fat treatments. The food intake by a mouse in the low-fat with PC group (LF-HPC) was the highest following by the food intake by a mouse in the low-fat group (LF-LPC). On the other hand, the food intake by a mouse in the high-fat with PC group (HF-HPC) was the lowest following by the food intake by a mouse in the high-fat group (HF-LPC). The reason is that the low-fat diet as described in **Table 3.2.2.2** had fewer calories per gram than the high-fat diet, 773.85 g of the high-fat diet had the same amount of calories, 4057 kcal, as 1055.1 g of the low-fat diet. Therefore, the mice in the low-fat groups needed to eat more than the mice in the high-fat groups to obtain the same amount of energy. The differences in food intake by cage between treatments over two weeks are shown in **Figure 3.3.2.** Two-way ANOVA showed that the intake of both of the high-fat groups (HF-LPC, HF-HPC) was significantly different from the intake of both of the low-fat groups (LF-LPC, LF-HPC) (fat: $p < 0.0001$, PC: NS, fat*PC: NS).

Table 3.3.2. The average food intake per mouse (g/mouse).

Treatment	Week 1	Week 2
LF +LPC	27.4	30.9
LF +HPC	30.6	37.5
HF +LPC	18.3	19.1
HF + HPC	17.1	17.6

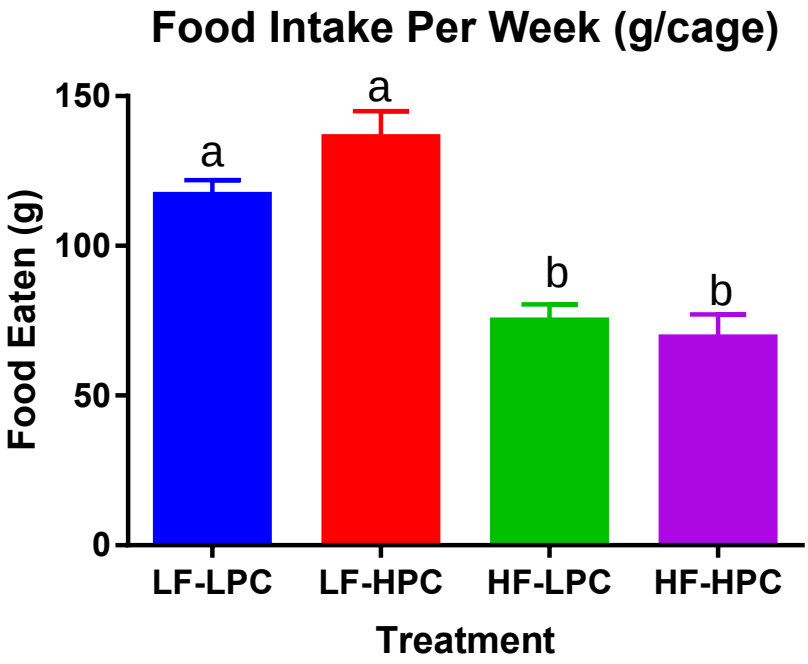

Figure 3.3.2. The lines represent calculated food intake values (g / cage of each treatment) over two weeks. Data displayed are mean ± SEM of n=12 mice/treatment. The four treatments are low-fat (LF-LPC), low-fat with PC(LF-HPC), high-fat(HF-LPC), and high-fat with PC (HF-HPC). Two-way ANOVA showed significant differences between the HF groups and the LF groups intake (alpha=0.05, fat: $p < 0.0001$).

3.3.3 Weight gain.

Weight gain during the study is displayed in **Figure 3.3.3.** The weight gain was consistent over time. When two-way ANOVA was applied to the percentage of weight gain overall (the first graph), the treatment results weren't significantly different. That was expected because the study was performed over only 15 days. Longer time might be required to observe significant differences in weight gain. The result from a 13-week mice study showed that mice fed HF diet didn't weigh significantly different from mice fed LF diet over the entire 13 weeks. However, the mice fed HF diet had a significantly higher percentage of body fat than the mice fed LF diet [77]. A previous two-week mice study by Goodrich *et al.* showed no significant differences between treatments in weight gain, which is another evidence for not observing significant differences in weights during short-period of time [78]. When applying two-way ANOVA to the weights over time (the second graph), the results showed that the weights of the high-fat with PC group (HF-HPC) were significantly different from the low-fat group (LF-LPC) at day 8 (fat: p=0.0285, PC: p=0.0411, fat*PC: NS). Otherwise, no more significant differences occur in weights.

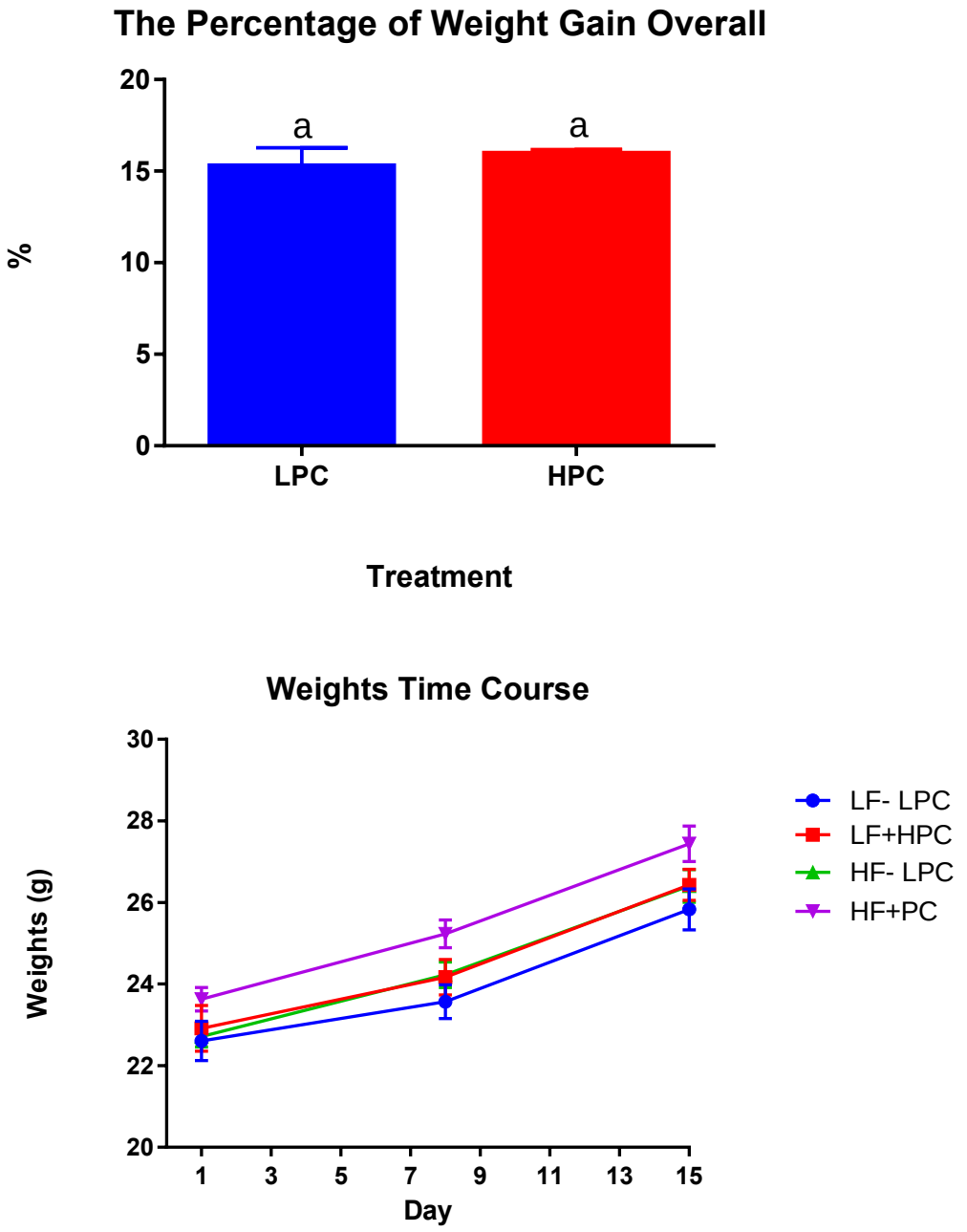

Figure 3.3.3. The bar graph shows the percentage of weight gain overall (%) among groups throughout the study. Data presented are the mean ± SEM of n=12 mice/treatment. The lines illustrate the increased rate of weights over time. The four treatments are low-fat (LF-LPC), low-fat with PC(LF-HPC), high-fat(HF-LPC), and high-fat with PC (HF-HPC). Two-way ANOVA displayed no significant differences in the percentage of weight gain overall. However, two-way ANOVA was applied to weight gain over time, and the results showed a significant difference between HF-HPC and LF-LPC at day 8 (alpha=0.05, fat: p=0.0285, PC: p=0.0411).

3.3.4 Body composition.

Fat and lean gain during the study is displayed in **Figure 3.3.4.** Body composition was performed twice, at the beginning and the end. Two-way ANOVA showed no significant differences between treatments. Slight differences were observed in fat gain between the two high-fat groups (HF-HPC, HF-LPC) though the only difference was the PC added. Similarly, the slight differences observed in lean gain between the two high-fat groups (HF-HPC, HF-LPC) and the two-fat groups (LF-HPC-LF-LPC) weren't expected. The change in body fat and lean was consistent over time. The low-fat group (LF-LPC) gained the least amount of fat, but the most amount of lean compared to the others. The high-fat group with PC (HF-HPC) gained the most amount of fat but lost the most amount of lean compared to the others. The other groups lost some body lean but gained body fat.

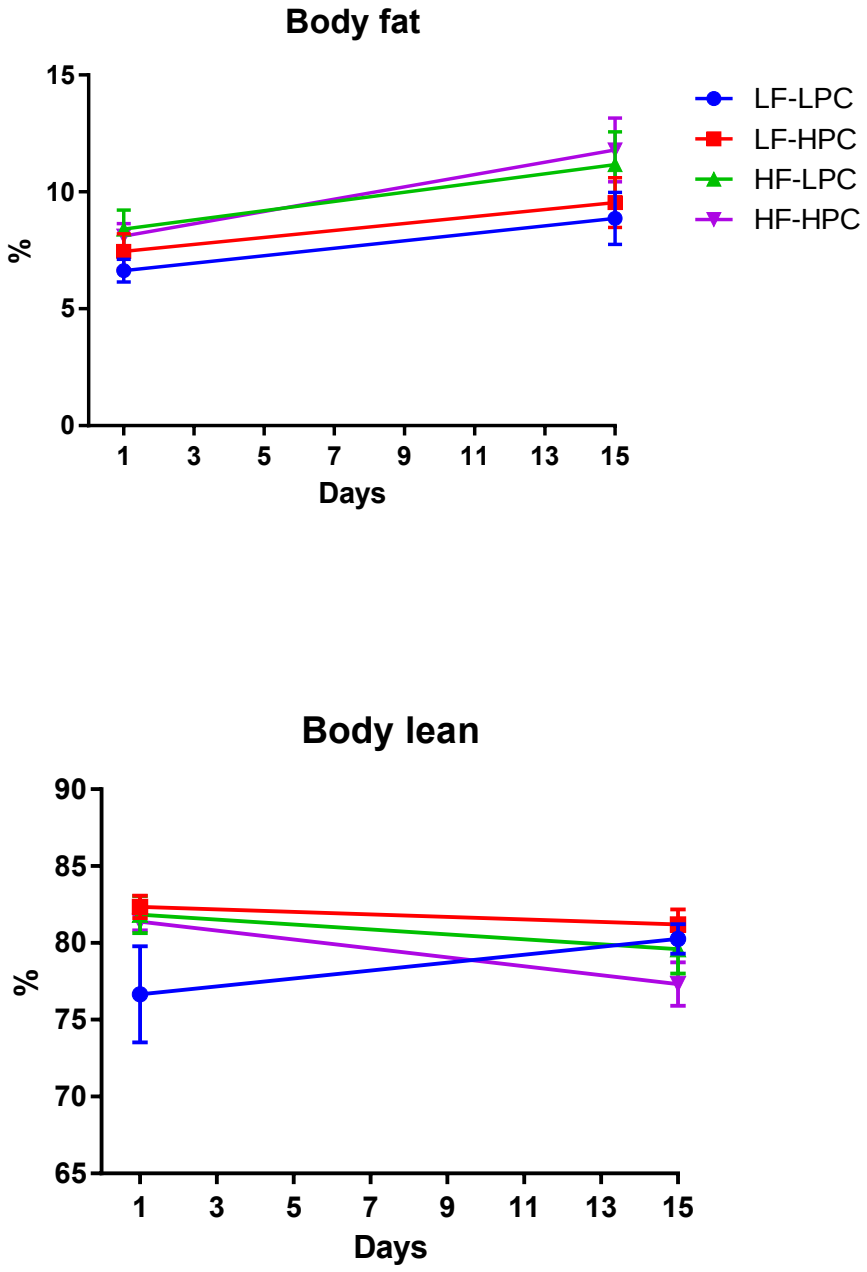

Figure 3.3.4 The first graph shows the total percentage of fat gained versus time (day 1,15). The second graph shows the total percentage of lean gained versus time (day 1,15). Data displayed are the mean ± SEM of n=12 mice/treatment. The four treatments are low-fat (LF-LPC), low-fat with PC(LF-HPC), high-fat(HF-LPC), and high-fat with PC (HF-HPC). Two-way ANOVA results showed no significant differences among groups.

3.3.5 Non-fasting blood result

Figure 3.3.5.1 displays the measurement of non-fasting plasma TMAO levels in treatments. It was expected to observe the highest concentrations of TMAO with the high-fat groups (HF-HPC, HF-LPC) as TMAO precursors are mostly found in high-fat foods [33] and a high-fat intake provides more cholesterol and phospholipids. However, the opposite happened. The results of the low-fat groups (LF-LPC, LF-HPC) showed higher concentrations of TMAO. After running two-way ANOVA, TMAO result showed that the low-fat with PC group (LF-HPC) was significantly different from all groups including the low-fat group (LF-LPC), and the low-fat group (LF-LPC) was significantly different from both of the high-fat groups (fat: $p < 0.0001$, PC: NS, fat*PC: $p=0.0266$). Otherwise, no statistically significant differences observed among treatments. These results showed that fat appeared to suppress the production of TMAO which is against a previous finding that a short-term high fat diet led to an increase in postprandial TMAO levels [1]. A first possible explanation would be that fat suppressed PC metabolism, the amount of choline that is released from PC. PC is a lipid. Therefore, when PC was added to the high-fat diet, other fat substances competed with it to be metabolized. If this suggestion is true, then PC wasn't the right choice to be used in this study. This result is evidence to use another TMA substrate that is not lipid such as choline in future studies. A second possible explanation would be that the composition of microbiota that associated with TMA production was higher in the mice fed the low-fat diet which means that a high-fat diet inhibited of TMAO production. However, previous evidence suggested that a high-fat diet changes the composition of microbiota increasing bacteria associated with TMA production such as *Firmicutes* [21,65]. The last possible explanation would be that the food intake had an impact on the results. The intake of the low-fat groups was higher than the food intake of the high-fat groups. Both of the fat-diets

contained two grams of choline bitartrate. As a result, the low-fat groups' data showed higher concentrations of TMAO. It was believed that the results could be better if choline bitartrate had been excluded from both of the diets. To test if the last possibility might be true, TMAO results were normalized (divided) by the average food intake per treatment. The result of the normalized data is displayed in **Figure 3.3.5.2.** The graph is approximately the same if compared to the original. Statistically, two-way ANOVA showed significant differences between the low-fat with PC group (LF-HPC) and the two high-fat groups (HF-HPC, HF-LPC) (fat: p= 0.0004, PC: NS, fat*PC: NS). However, the result of the low-fat group (LF-LPC) wasn't significantly different from the low-fat with PC group (LF-HPC) result. This outcome weakens the possibility of the impact of food intake on the results. Despite the fact that the mechanism by how fat and PC impact on the results was unclear, it was interesting to observe differences in TMAO levels between the LF groups.

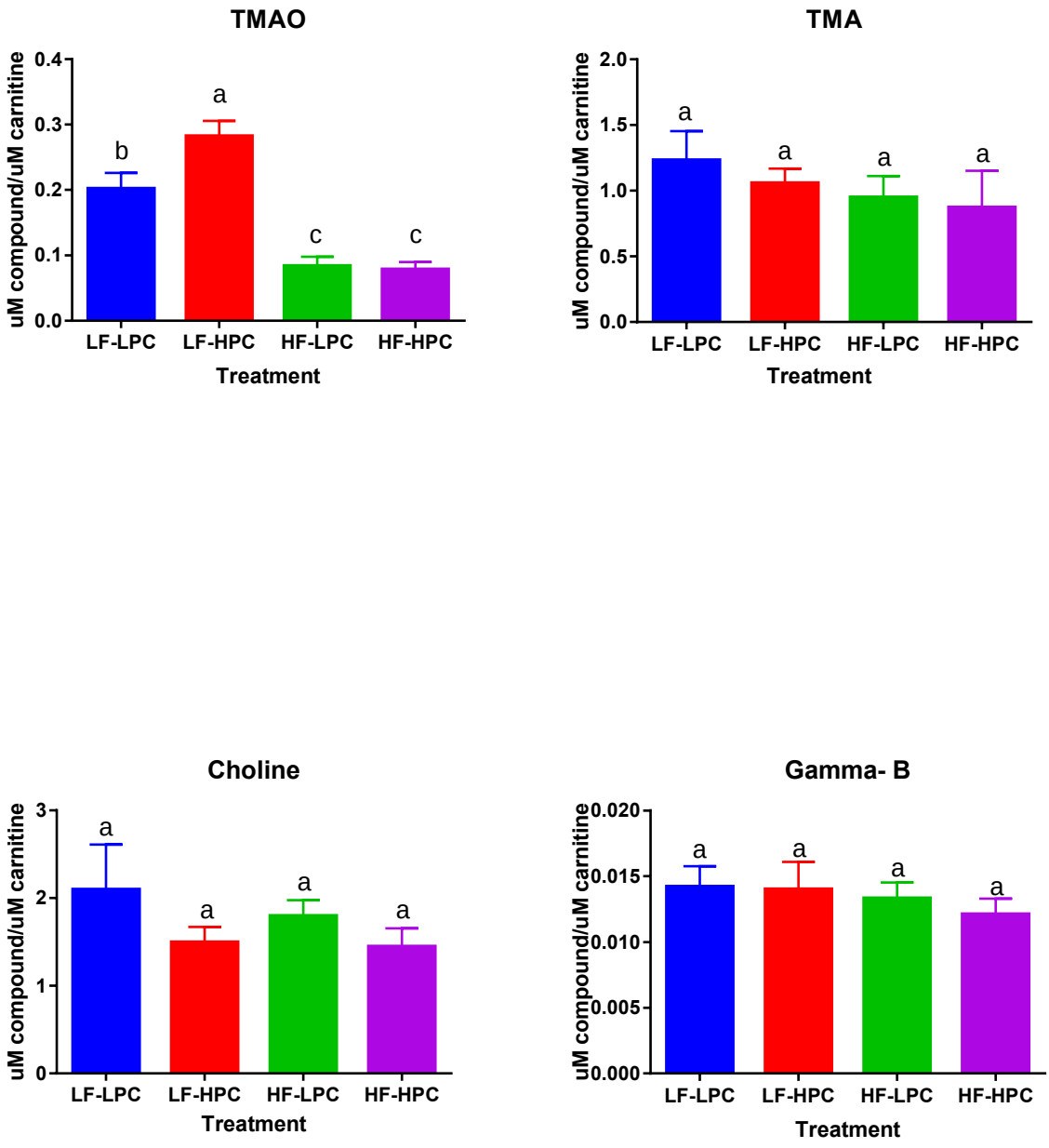

Figure 3.3.5.1 Bar graphs illustrate the different levels of TMAO, TMA, choline, and gamma-butyrobetaine between treatments. Data gained from the analyzed non-fasting blood. Data displayed are the mean ± SEM of $4 \leq n \leq 12$ mice/treatment. The four treatments are low-fat (LF-LPC), low-fat with PC(LF-HPC), high-fat(HF-LPC), and high-fat with PC (HF-HPC). Two-way ANOVA of TMAO results showed a significant difference between the low-fat and high-fat groups and between the two low-fat groups (alpha=0.05, fat: $p< 0.0001$, PC: NS, fat*PC: $p= 0.0266$)

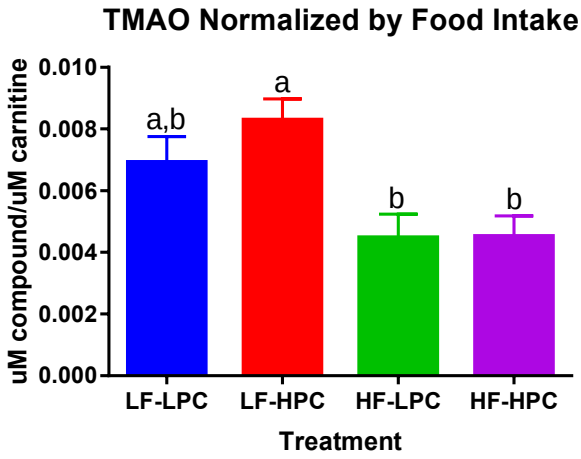

Figure 3.3.5.2 Bar graphs illustrate the different levels of TMAO normalized by the average food intake per cage in treatments. Data gained from the analyzed non-fasting blood. Data displayed are the mean ± SEM of $4 \leq n \leq 12$ mice/treatment. The four treatments are low-fat (LF-LPC), low-fat with PC(LF-HPC), high-fat(HF-LPC), and high-fat with PC (HF-HPC). Two-way ANOVA showed a significant difference between the low-fat with PC group and the two high-fat groups (alpha=0.05, fat: p= 0.0004, PC: NS, fat*PC: NS).

3.3.6 Fecal result

Fecal pellets were collected randomly from cages, so the results didn't represent mice individually. Therefore, extraction and quantification of TMA and its precursors were repeated three times to get more reasonable results and to reduce the variation in error bars. Replication wasn't available in non-fasting blood results because the amount of blood wasn't enough for more than one sample preparation. The results of the measured levels of fecal TMA and its precursors are displayed in **Figure 3.3.6.1.** It was expected to observe TMA in the results as it was released by the gut microbiota, but it wasn't expected to observe TMAO concentrations because they are usually made by the liver enzyme. The amount of choline was the same across groups, so it was expected to observe higher levels of TMA, and its precursors in the high PC groups (LF-HPC, HF-HPC) compared to the low PC groups (LF-LPC, HF-LPC), and in the high-fat groups compared to the low-fat groups. However, the results showed that the low PC

groups had higher TMA levels compared to the high PC groups. Similarly, TMAO, choline, and betaine concentrations were higher in the low PC groups compared to the high PC groups. Interestingly, the lines that represent the low PC groups went up by day 8, but then they went down by day 15. On the other hand, the lines that represent the high PC groups were slightly increasing, but consistently. As a result, TMA concentrations were higher by day 15 in the high PC groups compared to the low-fat group (LF-LPC). In general, TMA, choline, and betaine levels weren't as high as they were expected to be as we have seen some evidence for observing TMA levels in human feces in other studies [12, 54]. Statistically, two-way ANOVA showed that choline levels were significantly different between the LF-LPC and both of the high PC groups (LF-HPC and HF-HPC) at day 8 (fat: NS, PC: p= 0.0142, fat*PC: NS). Interestingly, the amount of choline was higher in the LF-LPC group than the amount of choline in the two high PC groups. There was a main effect of PC on TMA and TMAO levels at day 8 (p= 0.0202, 0.0486, respectively), but no significant differences observed between individual treatments. Similarly, there was a main effect of fat on gamma-butyrobetaine levels at day 15 (p= 0.0451), but no significant differences observed between individual treatments. There was an interaction between fat and PC on choline levels at day 1 (p= 0.0248), but no significant differences observed between individual treatments. Looking at gamma-butyrobetaine, the results of all treatments had the same pattern. Similarly, the results of almost all treatments showed no detectable carnitine levels, except the low-fat with PC group. It had small amounts of carnitine at day 8. A possible explanation for not observing microbial activity in fecal TMA levels would be that whether the bacteria associated with choline metabolism was present in the small intestine, but not in the colon or that the bacteria was present, but TMA lyase used by the bacteria to release TMA was missing. Bile acids were shown to decrease the number of microbiota in the

small intestine during circulation of bile acids from the liver into the bile [52]. Another possible explanation would be that the enzymes, phospholipases or glycerophosphoryldiester phosphodiesterases, needed to release choline from PC were not available in the colon. To clarify if the food intake had an impact on the results, data were normalized by the average food intake per treatment, see **Figure 3.3.6.2.** The results were found to be similar to the original. One minor difference is that the high-fat group (HF-LPC) had higher TMAO and TMA concentrations than the low-fat group (LF-LPC) when data were normalized. When two-way ANOVA was applied, the results of gamma-butyrobetaine were significantly different at day 8 between the LF-HPC and both of the high-fat groups (fat: p=0.0006, PC: NS, fat*PC: NS). Additionally, the results of gamma-butyrobetaine levels at day 15 were significantly different between LF-HPC and HF-LPC (fat: p= 0.0019, PC: NS, fat*PC: NS). There was a main effect of PC on TMA levels at day 8 (p= 0.0361), but no significant differences observed between individual treatments. Similarly, there was a main effect of fat on TMAO levels at day 1 (p= 0.0108), but no significant differences observed between individual treatments. These unexpected results might be explained well if the impact of enzymes on the liver and the gut is explained.

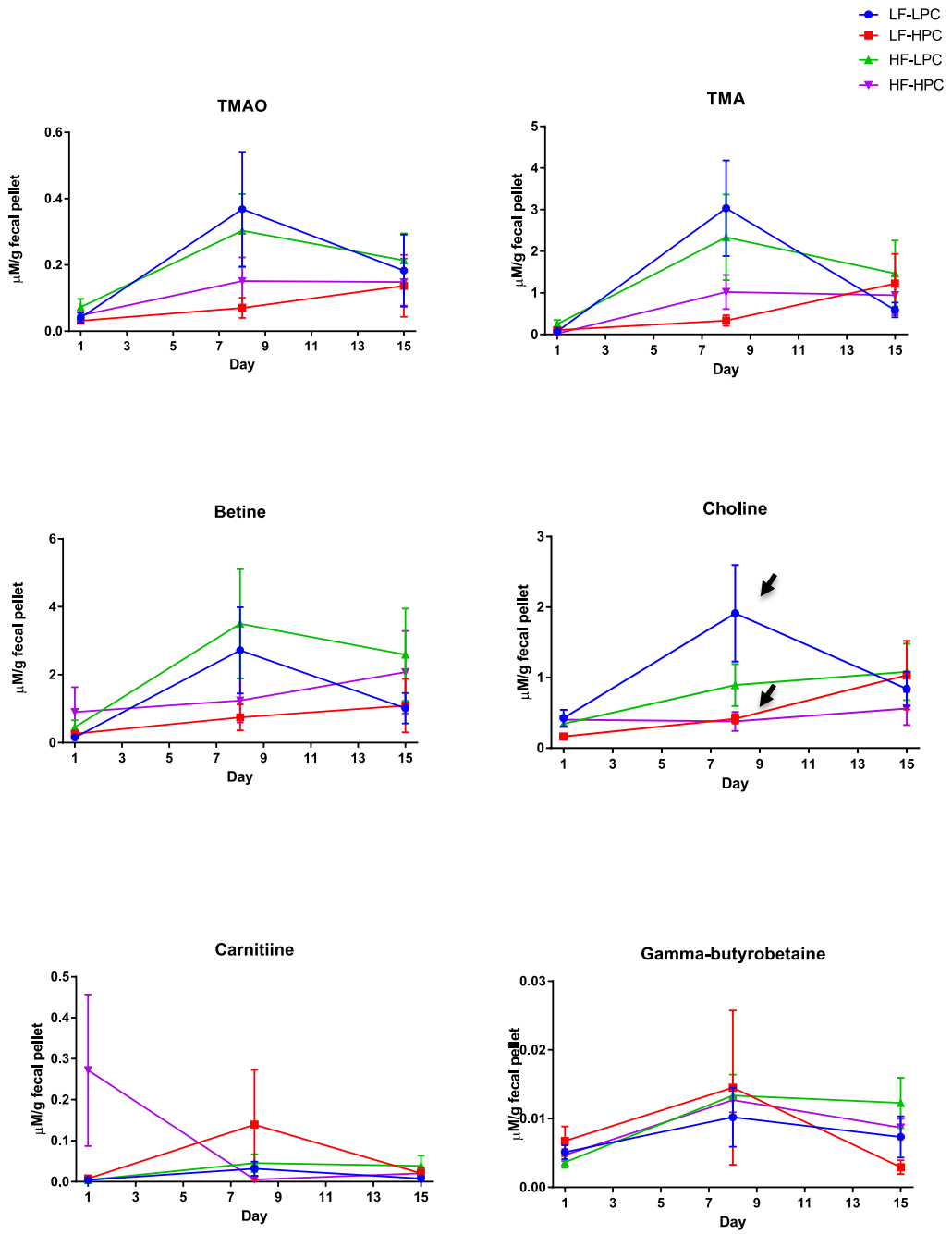

Figure 3.3.6.1 Illustration of TMAO, TMA, choline, carnitine, betaine, and gamma-butyrobetaine concentrations (µM/ g of fecal pellet) among treatments. The four treatments are low-fat (LF-LPC), low-fat with PC(LF-HPC), high-fat(HF-LPC), and high-fat with PC (HF-HPC). Data displayed are the mean ± SEM of n = 3 cage /treatment (replicate = 3). Two-way ANOVA showed significant differences in choline levels between the LF-LPC group and the two PC groups at day 8 (alpha= 0.05, fat: NS, PC: p= 0.0142, fat*PC: NS).

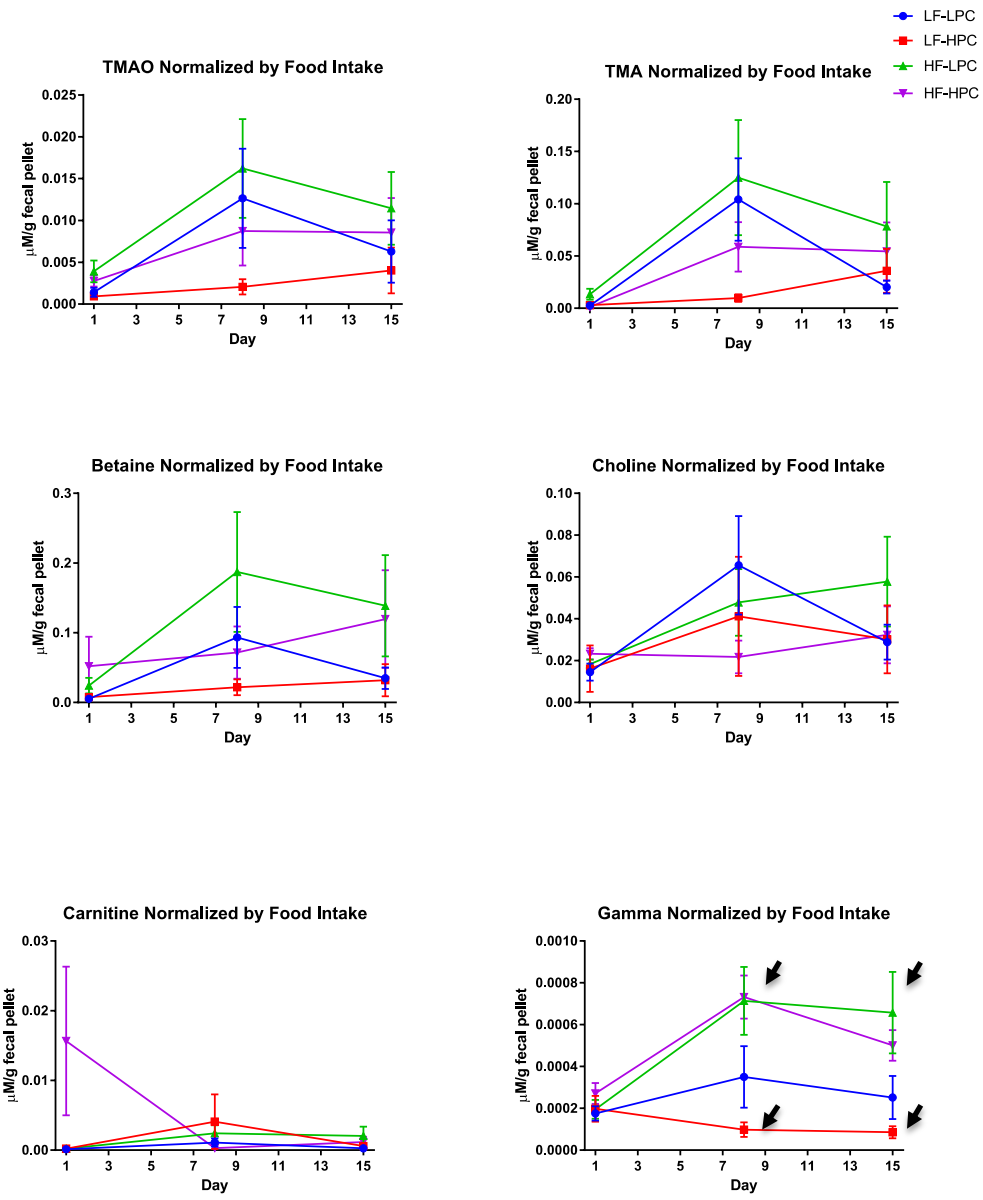

Figure 3.3.6.2 Illustration of TMAO, TMA, choline, carnitine, betaine, and gamma-butyrobetaine concentrations (µM/ g of fecal pellet) among treatments after they were normalized by the average food intake. The four treatments are low-fat (LF-LPC), low-fat with PC(LF-HPC), high-fat(HF-LPC), and high-fat with PC (HF-HPC). Data displayed are the mean ± SEM of n = 3 cage /treatment (replicate = 3). Two-way ANOVA showed significant differences in gamma-butyrobetaine levels at day 8 between LF-HPC group and the two high-fat groups (fat: p=0.0006, PC: NS, fat*PC: NS). and at day 15 between LF-HPC group and HF-LPC group (fat: p= 0.0019, PC: NS, fat*PC: NS).

3.4 Conclusion

The study aim was indirectly measuring of microbiota adaptation to different dietary patterns in mice. The mice were maintained for two weeks on different treatments that were either a high-fat or low-fat diet with or without PC. Then, a high-fat high-PC meal challenge was provided to the four treatments after the two weeks. Quantification of fecal TMA, fasting and postprandial TMAO, and non-fasting TMAO levels were performed on LC-MS/MS. After measuring of non-fasting blood TMAO levels, the combination of low-fat with or without PC showed a microbial activity that resulted in increased TMAO levels. These results showed that fat appeared to suppress the production of TMAO. It was unclear whether fat suppressed the metabolism of PC or whether gut microbiota associated with TMA production was higher in the mice fed a low-fat diet than in the mice fed a high-fat diet. Also, it was unclear why TMA and its precursors weren't observed in high concentrations in the blood. The average food intake across treatments might have an impact on increased levels of TMAO as it was observed to be higher with the low-fat groups. The mechanism by how PC levels influenced circulating TMAO levels is still unclear. The microbial adaptation to the different treatments wasn't observed in the measurement of fecal TMA levels. The impact of PC on fecal TMAO and TMA levels wasn't clear. It was unclear whether the bacteria that can metabolize choline was missing in the colon or whether TMA lyase was missing. The results of measuring fasting and postprandial plasma TMAO levels before and after the meal challenge weren't available because the blood drops weren't enough to be quantified with LC-MS. Further studies are needed to explain what happened.

Chapter 4: Research Limitations

During the three gavage days, we had some difficulties applying the meal challenge containing 3.33g of corn oil, 3.33 of cornstarch, 3.33 casein, and 0.75 g soybean L-α-Lecithin per 1 kg body weight (as originally planned). The mixture was thick, so it was difficult to be pushed out through syringes. Instead, a meal containing 9.99 g of corn oil with 0.75 g soybean L-α-Lecithin per 1 kg body weight was provided to the mice. Also, ethyl chloride was supposed to be used as pre-anesthetic to nip the effect of tail tip snips, but it wasn't available in the vivarium. Therefore, alcohol with ice replaced the use of ethyl chloride. Unfortunately, the results of the blood collected over 12 hours before and after the acute meal weren't available. The blood drops were not enough to be quantified by LC-MS. The use of rats with catheters in future studies is needed in ordered to get enough blood. Additionally, both of the diets (high-fat diet and low-fat diet) contained 2 % (by the weight of diet) of choline bitartrate, TMA containing substrate. The amount of choline bitartrate in the diets was higher if compared to the amount of PC (0.5% by the weight of diet) added to the high PC treatments (HF-HPC, LF-HPC). The results showed that the average food intake by the low-fat groups was higher than the average food intake by the high-fat groups. As a result, the low-fat groups' data showed higher concentrations of TMAO than the high-fat groups. Therefore, the use of PC in the diets didn't show the expected results. The effect of PC could be observed better if choline bitartrate had been excluded from both of the diets or if the amount of PC was higher than the amount of choline bitartrate relatively. These results suggest the use of other TMA containing substrates such as choline and L-carnitine instead of PC. Though dietary choline found mostly as PC, the impact of choline on TMA formation is larger compared to PC. The results of urinary excretion of TMA in humans after the intake of different types of choline supplementations showed that the intake of choline led to a

large increase in TMA production, but PC led only to a small increase. This increase was even a result of consuming lecithin that was contaminated with TMA, so cleaning up lecithin from this contamination, by removing TMA, resulted in no increase in urine extraction of TMA [35]. Similarly, in a previous study forty-six different foods were provided to humans. By looking at the urinary extraction of TMA and TMAO, the authors found that 60% of free choline and 30% carnitine were converted to one of these two products, but they didn't find any levels of these two products in the urinary extraction after consuming either PC or betaine [36]. Based on that, the use of choline or L-carnitine in future studies instead of PC is recommended.

Chapter 5: Summary

TMAO levels were found to be associated with atherosclerosis in humans. TMAO production from dietary intake of high TMA containing substrates such as L-carnitine, choline, and PC is dependent on the microbiota adaptation and FMO3 expression. The consumption of a fish meal increases of circulating TMAO levels within 15 min of the consumption leading to suggest that TMAO is directly absorbed from the gut without undergoing the microbiota contribution. Increasing of fat intake might facilitate TMAO formation by providing more TMA precursors, inducing FMO3 expression, and manipulating the composition of the microbiota. Still, it wasn't clear if dietary intake of PC is sufficient to promote TMAO formation or if a high-fat intake is also required. We aimed to address the impact of different dietary patterns on circulating TMAO and fecal TMA levels by providing the mice with four different treatments that were either high-fat or low-fat with or without PC. The hypothesis was that TMAO would be increased after consuming both a high-fat diet and a high PC diet independently, with expected synergy between the two factors. The study aim was: determine the impact of a high-fat intake and a high PC intake individually or together on increased levels of TMAO. The results of measuring non-fasting circulating TMAO levels showed a microbial activity that resulted in increased TMAO levels. However, the microbial activity wasn't observed in the measurement of fecal TMA levels. The non-fasting blood TMAO results showed that the high-fat diet appeared to inhibit TMAO production. The combination of low-fat with or without PC led to higher increase in TMAO levels than the combination of high-fat with or without PC. Fecal TMA levels weren't observed as high as it was expected. Statistically, TMA levels weren't significantly different among treatments. As a result, our hypothesis was rejected. Future work addressing the gene expressions of enzymes on the gut and the liver is needed to explain what happened. In later

studies, the use of different TMA containing substrates such as choline and rats with catheters are recommended to clarify the mechanism by which fat and TMA containing substrate intake increases TMAO levels.

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Appendices

Appendix A: Food intake

Table 5.1. Mice pilot study food log

Diet			Experimental + control (high-fat or low-fat diets with or without PC)											
Week			Week 1			Week 1			Week 2			Week 2		
Day on			Monday (Jun 5)			Friday (Jun 9)			Monday (Jun 12)			Friday (Jun 16)		
Day off			Friday (Jun 9)			Monday (Jun 12)			Friday (Jun 16)			Monday (Jun 19)		
Treatment	Cage	# of mice in cage	Food Added (g)	Food Left (g)	Eaten (g)	Food Added (g)	Food Left (g)	Eaten (g)	Food Added (g)	Food Left (g)	Eaten (g)	Food Added (g)	Food Left (g)	Eaten (g)
(LF +LPC)	1	4	80.3	30.56	49.7	60.03	9.6	50.4	100.1	33.4	66.6	100.1	48.5	51.4
	2	4	80.6	14.5	66.1	60	5	55	100	26.6	73.4	100.1	42.3	57.8
	3	4	80.1	17.78	62.9	60.06	15	45.06	100.1	39.7	60.4	100.1	39.3	60.8
(LF +HPC)	1	4	80 +20.2	20	80.2	80.1	34	46.1	100.1	4.6	95.5	100.1	29	71.1
	2	4	80.7 +20	28	72.7	80	25	55	100 +20.1	30.2	89.9	100.1	31	69.1
	3	4	80 +20.2	28.8	71.4	80.06	38	42.06	100.15	21.3	78.85	100	55	45
(HF +LPC)	1	4	81.5	32.4	49.1	60.01	34	26	100	36.3	63.7	100	67	33
	2	4	80	28.67	51.9	60.06	33	27	100	58	42	100	81	19
	3	4	80	41.9	38	60.05	32	28	100.1	59.5	40.6	100.1	69	31.1
(HF + HPC)	1	4	80.3	40	40.3	60.05	41	19.05	100.1	67.4	32.7	100.1	56	44.1
	2	4	81.3	50.5	30.8	60.09	37	23.09	100	66.2	33.8	100	75	25
	3	4	81.6	25.9	55.7	60.05	24	36.05	100	54	46	100.1	71	29.1

Appendix B: Weight gain

Table 5.3. Mice pilot study weight log

			Weight (g)		
Treatment	Cage	Color	D 1[a] (Jun 5)	D 8 (Jun 12)	D 15 (Jun 19)
HF + HPC	1	Red	23.6	24.8	27.5
		Green	25.2	27.5	31
		Blue	24	26.1	28.3
		No-color	23.45	25.2	27
	2	Red	24.7	24.8	28
		Green	24.6	25.4	26.4
		Blue	23.15	24.7	28
		No-color	22.7	23.1	25.3
	3	Red	22	24.2	26.1
		Green	23.9	26.1	26.8
		Blue	24.1	26.6	28.7
		No-color	22.1	24.3	26.2
LF +HPC	1	Red	22.85	23.7	26.5
		Green	25.45	26.2	27.3
		Blue	19.2	21.6	23.8
		No-color	22.5	24	25.8
	2	Red	24.6	25.2	27.5
		Green	22.8	24.7	27.8
		Blue	19.5	21.1	24.1
		No-color	24.2	24.3	26
	3	Red	22.5	24	26.5
		Green	23.1	24.5	27.2
		Blue	25.2	25.7	27.5
		No-color	23.1	25	27.2
HF +LPC	1	Red	22.5	23.8	26.2
		Green	24.1	26.1	28.5
		Blue	22.1	24.3	26.7
		No-color	23.7	25.83	29.3
	2	Red	22.4	23.5	25.5
		Green	23	24	25.7
		Blue	22.75	24.8	26.7
		No-color	21.15	22.5	24
	3	Red	21.6	23.4	25.8
		Green	22.6	23	25.6
		Blue	23.85	25.1	25.9
		No-color	22.8	24.4	27
LF +LPC	1	Red	24.2	24.6	27.1
		Green	22.8	23.5	27.2
		Blue	25.6	27	29.4
		No-color	24.3	24.8	26.85
	2	Red	22.6	23.8	25.3
		Green	21.4	23.25	25
		Blue	22.2	22.9	26.2
		No-color	22.35	23.3	25.5
	3	Red	23.8	23.6	26.2
		Green	21.3	22	23.2
		Blue	21.25	22.6	24.8
		No-color	19.5	21.5	23.2

a: days on treatment.

Appendix C: Body Composition

Table 5.5. Mice pilot study body composition.

Treatment	Cage	Color	Fat (%) D 1	Fat (%) D 15	Fat gain %	Lean (%) D 1	Lean (%) D 15	Lean gain %
LF-LPF	1	Red	6.9	10.1	3.2	73	79.5	6.5
		Green	6.4	8.7	2.3	84.3	78.5	-5.8
		Blue	9.3	18.3	9	56.7	73.6	16.9
		Nan	4.6	10.2	5.6	62.5	82.3	19.8
	2	Red	6.2	3.1	-3.1	83.5	84.8	1.3
		Green	6.7	6.3	-0.4	84.5	81.4	-3.1
		Blue	5.6	6.7	1.1	82.2	81.6	-0.6
		Nan	6.3	5.1	-1.2	59.7	83.2	23.5
	3	Red	10.4	12.1	1.7	80.9	75.4	-5.5
		Green	6.2	7.2	1	84.6	80.3	-4.3
		Blue	4.5	9.7	5.2	82.7	78.6	-4.1
		Nan	6.4	8.9	2.5	85.2	83.8	-1.4
LF-HPC	1	Red	9.1	10.5	1.4	82.4	80.5	-1.9
		Green	9.2	9.5	0.3	80	77.3	-2.7
		Blue	9.4	4.2	-5.2	83.7	85.3	1.6
		Nan	6.8	7.2	0.4	83.1	82.6	-0.5
	2	Red	11.7	16.4	4.7	76.5	76.6	0.1
		Green	5	10.4	5.4	82.5	80.9	-1.6
		Blue	3	6.9	3.9	85.3	87.3	2
		Nan	3.7	6.7	3	83.6	83.2	-0.4
	3	Red	9.5	13.9	4.4	82.3	77.8	-4.5
		Green	8.6	14.2	5.6	79.8	77.4	-2.4
		Blue	7.6	8.3	0.7	83.4	83.1	-0.3
		Nan	5.9	6.3	0.4	85.6	82.4	-3.2
HF-LPF	1	Red	8.9	10.8	1.9	80.5	75.6	-4.9
		Green	11.5	15.9	4.4	81	74.6	-6.4
		Blue	8.6	15.4	6.8	81.9	77.8	-4.1
		Nan	10.9	17.8	6.9	72.2	73	0.8
	2	Red	8.3	12	3.7	83.1	80.1	-3
		Green	4.1	6.3	2.2	85	86.8	1.8
		Blue	11	10.4	-0.6	81.1	77.2	-3.9
		Nan	5.6	3.5	-2.1	86.5	86.8	0.3
	3	Red	10.5	15.4	4.9	77.5	76.2	-1.3
		Green	4.3	4.1	-0.2	87.1	87.3	0.2
		Blue	5.6	7.6	2	85.9	85.5	-0.4
		Nan	11.5	14.9	3.4	80.3	74.2	-6.1
HF-HPC	1	Red	8.1	12.6	4.5	80.2	76.2	-4
		Green	9.8	14.8	5	80.7	71.2	-9.5
		Blue	7	7	0	85.4	82.4	-3
		Nan	4	4.3	0.3	83.9	86.8	2.9
	2	Red	10.8	7.4	-3.4	81.2	79.2	-2
		Green	6.8	5.7	-1.1	81.6	84.1	2.5

		Blue	7.5	14.7	7.2	79.2	71.6	-7.6
		Nan	7.5	10.1	2.6	82.3	77.2	-5.1
	3	Red	7.7	14.4	6.7	81.1	74.3	-6.8
		Green	8.3	14.5	6.2	81.1	76.1	-5
		Blue	10.9	17.7	6.8	81.9	74.9	-7
		Nan	8.9	18.4	9.5	78	73.8	-4.2