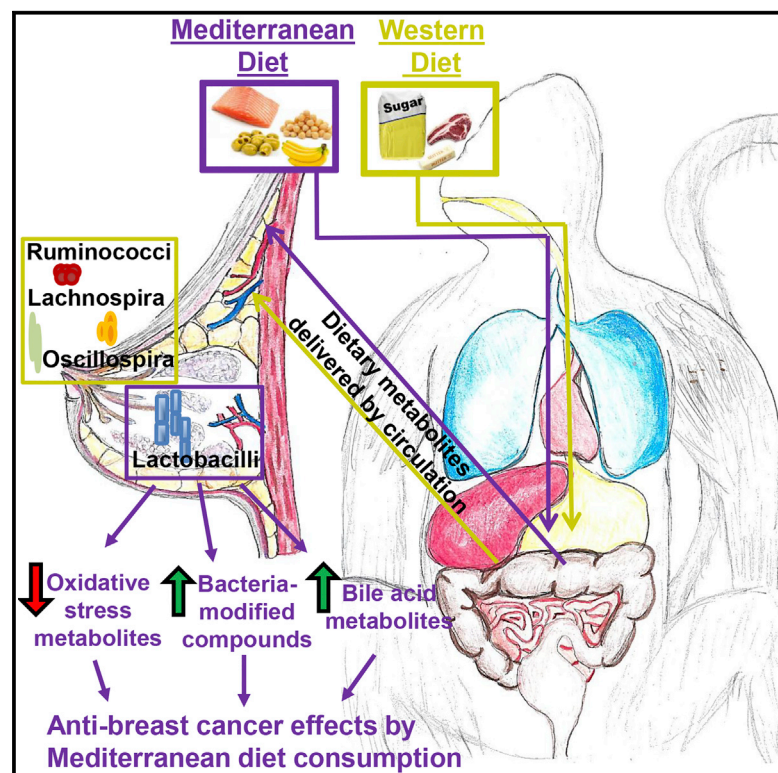


Consumption of Mediterranean versus Western Diet Leads to Distinct Mammary Gland Microbiome Populations

Graphical Abstract



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In Brief

Using a non-human primate model of women's health, Shively et al. demonstrate that diet plays a critical role in determining microbiota populations in tissues outside the gut, such as the mammary gland. These microbial populations modulate localized bile acid and bacterial-modified metabolites to potentially influence anticancer signaling pathways.

Highlights

- Diet modulates mammary gland microbiota populations in a non-human primate model
- Consumption of Mediterranean diet elevates mammary gland *Lactobacillus* abundance
- Mediterranean diet increases breast bile acid and bacterial-modified metabolites
- Consumption of Mediterranean diet decreases reactive oxygen species metabolites



Consumption of Mediterranean versus Western Diet Leads to Distinct Mammary Gland Microbiome Populations

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SUMMARY

Recent identification of a mammary gland-specific microbiome led to studies investigating bacteria populations in breast cancer. Malignant breast tumors have lower *Lactobacillus* abundance compared with benign lesions, implicating *Lactobacillus* as a negative regulator of breast cancer. Diet is a main determinant of gut microbial diversity. Whether diet affects breast microbiome populations is unknown. In a non-human primate model, we found that consumption of a Western or Mediterranean diet modulated mammary gland microbiota and metabolite profiles. Mediterranean diet consumption led to increased mammary gland *Lactobacillus* abundance compared with Western diet-fed monkeys. Moreover, mammary glands from Mediterranean diet-fed monkeys had higher levels of bile acid metabolites and increased bacterial-processed bioactive compounds. These data suggest that diet directly influences microbiome populations outside the intestinal tract in distal sites such as the mammary gland. Our study demonstrates that diet affects the mammary gland microbiome, establishing an alternative mechanistic pathway for breast cancer prevention.

INTRODUCTION

Diet has been extensively studied as a modifiable component of lifestyle that could influence breast cancer development. The Mediterranean diet is considered to be one of the healthiest dietary habits (Willett et al., 1995). Mediterranean dietary patterns are characterized by consumption of cereals (preferably as whole grains), legumes, nuts, vegetables and fruits, fish or seafood, white meat and eggs, and moderate to small amounts of

poultry and dairy products. The principal source of dietary lipids in the Mediterranean diet is olive oil.

It has been suggested that a Mediterranean dietary pattern can protect against diabetes, cardiovascular disease, and cancer (Haeghele et al., 1994; Pelucchi et al., 2009). Reported consumption of a Mediterranean diet pattern was associated with lower breast cancer risk for women with all subtypes of breast cancer, and a Western diet pattern was associated with greater risk (Castelló et al., 2014). Protective effects of Mediterranean diet pattern on breast cancer risk were also observed in a recent prospective cohort study (Buckland et al., 2013) and a randomized, single-blind, controlled field trial in which participants were randomly allocated to a Mediterranean diet supplemented with either extra-virgin olive oil or mixed nuts or a control diet and advised to reduce dietary fat (Toledo et al., 2015). Associations between dietary intake and cancer may not be ascribed to a single nutrient but rather to multiple nutrients and foods and their synergistic effects. Thus, assessing diet as a whole, on the basis of overall dietary patterns, may provide more useful information on the role of diet in breast cancer risk than a single-nutrient approach. However, inference from population studies is difficult because of measurement error in dietary assessment that arises from dietary data being self-reported, often retrospective, and collected using food frequency questionnaires that require many assumptions (i.e., recipes, food composition tables) to derive nutrient and food intakes.

Diet is also recognized as a major component shaping the gut microbiome. Distinct patterns of gut microbiota composition are associated with the habitual consumption of animal fats, high-fiber diets, and vegetable-based diets (Cotillard et al., 2013; David et al., 2014; Schnorr et al., 2014; Wu et al., 2011). Adherence to a Mediterranean diet decreased *Ruminococcus* and increased fecal *Lachnospira* and *Prevotella* in fecal samples, compared with samples from people consuming an omnivore diet (De Filippo et al., 2016). However, dietary influences on the microbiome in other tissue or organ sites other than the gut are undetermined.

A mammary gland-specific microbiome has been identified (Urbaniak et al., 2014). Mammary gland samples obtained from women undergoing lumpectomies, mastectomies, or breast



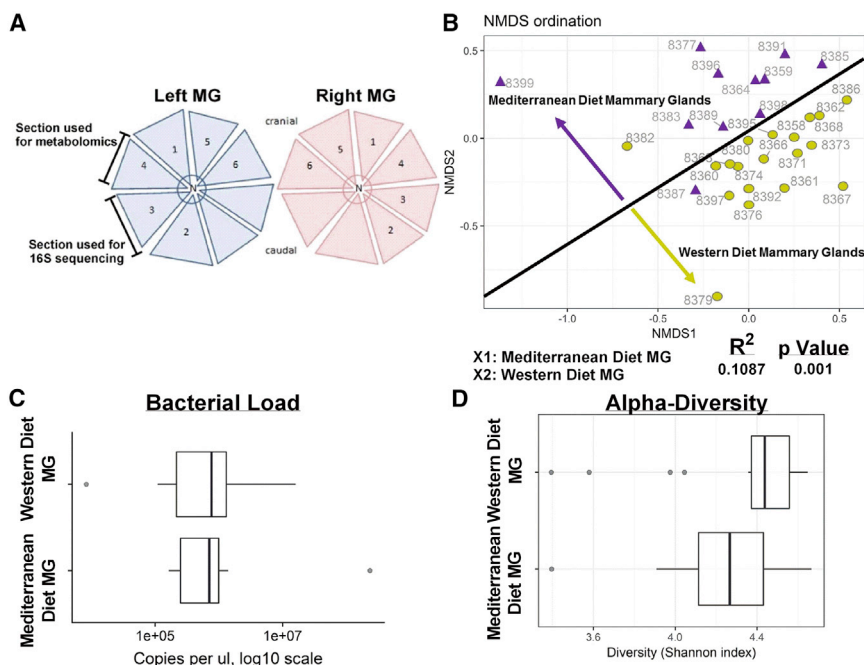


Figure 1. Habitual Diet Shifts Mammary Gland Bacterial Populations with No Effect on Quantity or Diversity

(A) Diagram of mammary gland sections aseptically obtained and used for analysis.

(B) Non-metric multidimensional scaling (NMDS) analysis to visualize microbiome similarities in ordination plots. Western diet 16S samples ($n = 19$) shown as mustard green circles and Mediterranean diet 16S samples ($n = 11$) shown as purple triangles. Variation in community structure was assessed with permutational multivariate ANOVA (PERMANOVA) with treatment group as the main fixed factor. Microbiota from Western diet-fed monkey mammary gland samples significantly varied from Mediterranean diet-fed monkey mammary gland microbiota ($R^2 = 0.1087$, $p = 0.001$).

(C) Bacterial load was determined via qPCR targeting the V4 region of the 16S gene. Results are expressed in copy number per microliter of DNA extract. No significant differences were observed by diet groups.

(D) Alpha diversity was estimated with the Shannon index on raw OTU abundance tables after filtering out contaminants. No significant differences were observed by diet groups. Error bars show the min to max distribution.

reduction procedures living in Canada or Ireland showed distinct genus taxa differences: mammary gland samples from Canadian women displayed a high proportional abundance of *Bacillus*, *Acinetobacter*, *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus*, *Propionibacterium*, and *Prevotella*. Mammary gland samples from Irish women displayed a more than 3-fold higher abundance of *Enterobacteriaceae*, a 2-fold higher abundance of *Staphylococcus*, high *Listeria welshimeri*, a 2-fold higher abundance of *Propionibacterium*, and similar proportional abundance of *Pseudomonas* compared with Canadian women's breast samples (Urbaniak et al., 2014). Whether the observed taxa differences or abundance differences were due to differences in BMI or obesity is unknown, because those data were unreported in the study. In another study investigating the microbiome of mammary gland tissue obtained from patients with benign tumors or malignant breast cancers, breast tissue from those with malignant breast cancer had decreased *Lactobacillus* (Hieken et al., 2016), suggesting mammary tissue dysbiosis as a possible driver of breast cancer.

To investigate the potential of diet pattern to modify breast tissue-specific microbiota populations, we obtained mammary gland samples from female *Macaca fascicularis* monkeys fed a Western diet or a Mediterranean diet for 31 months. This species is a well-established model of women's health, with considerable use in the study of breast cancer risk (Dewi et al., 2013; Shively et al., 2004; Stute et al., 2006, 2012; Wood et al., 2006, 2007a, 2007b, 2007c). Our present study demonstrates that diet modulates mammary gland-specific microbiota. Consumption of a Mediterranean diet was associated with 10-fold higher breast tissue *Lactobacillus* abundance and altered breast tissue bile acid metabolite levels and bacterial-processed bioactive compounds, clearly demonstrating a dietary contribution to

mammary gland health and homeostasis with implications for breast cancer prevention.

RESULTS

Female monkeys (*M. fascicularis*) were fed a Western diet or a Mediterranean diet for 31 months (see Table S1 for diet details). Mammary gland tissue sections (locations of tissue samples harvested for analyses are shown in Figure 1A) were used for 16S sequencing. We sequenced 16S (V4 region) genes on an Illumina MiSeq. We obtained 9.2096×10^4 high-quality bacterial reads. The final dataset had 1,470 operational taxonomic unit (OTUs) (including those occurring once with a count of 1) and a read range of 560 and 6,292. We excluded samples with fewer than 500 reads; a more typical cutoff is 1,000 reads. The number is arbitrary, but it is sufficient to reveal the most important gradients in microbial composition, even in high-diversity settings such as the gut. In breast tissue, where microbial diversity is lower, we used a lower cutoff to be sufficient.

We summarized OTU abundances with the Bray-Curtis index and performed a non-metric multidimensional scaling (NMDS) analysis to visualize microbiome similarities in ordination plots (Figure 1B). Formal significance testing was achieved with permutational ANOVAs. Mediterranean diet consumers had markedly different mammary gland microbiota populations compared with mammary glands from monkeys consuming a Western diet, resulting in two distinct microbiota populations based on dietary consumption. Diet had no effect on microbial load (Figure 1C) or alpha diversity (Figure 1D).

High-quality reads were classified using Greengenes as the reference database. We obtained consensus taxonomy for each OTU. We then aggregated OTU abundances into

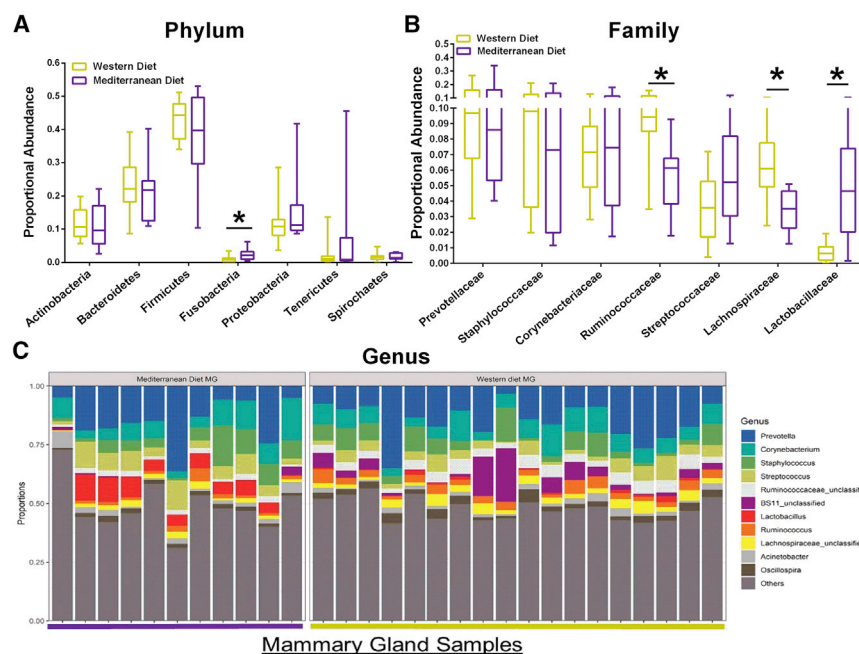


Figure 2. Diet Modulates Mammary Gland Microbiota Clustering and Taxonomic Profiles

(A) Taxonomic profiles of the mammary gland tissue from monkeys consuming a Western or Mediterranean diet at phylum level. * $p < 0.05$. (B) Community composition between Western diet-fed and Mediterranean diet-fed monkey mammary gland samples at family level. * $p < 0.05$. (C) Relative abundance of bacterial genera in different breast tissue samples is visualized by bar plots. Each bar represents a subject and each colored box a bacterial taxon. The height of a color box represents the relative abundance of that organism within the sample. “Other” represents lower abundance taxa. $n = 11-18$, * $p < 0.05$. Error bars show the min to max distribution.

(Holmes et al., 2012; Zhang and Davies, 2016). Multiple metabolomic studies using germ-free and conventionally housed mice along with antibiotic-treated mice have identified metabolites regulated by host bacterial populations. We observed higher levels of many of these bacteria-

processed metabolites in the mammary glands of Mediterranean diet-fed monkeys relative to metabolite levels in mammary glands of Western diet-fed monkeys (Figure 5). Consumption of Mediterranean diet was associated with significant elevations of hippurate (Figure 5A), p-cresol glucuronide (Figure 5B), 3-hydroxy-3-phenylpropionate (Figure 5C), cinnamoylglycine (Figure 5D), 3-indoxyl sulfate (Figure 5E), indole propionate (Figure 5F), indolin-2-one (Figure 5G), and trimethylamine N-oxide (Figure 5H) compared with Western diet. Mammary gland levels of the parent amino acids from which the bioactive microbial-derived compounds are derived (phenylalanine, tyrosine, and tryptophan) were not significantly altered between dietary groups in the mammary glands (Figure S2). Analyses of circulating plasma levels of bacterial-processed metabolites indicate differential regulation of bacterial-modified compounds when comparing regulated metabolites in the mammary gland with bacterial-modified metabolites in plasma samples. There were no diet-related differences in levels of phenylalanine-derived bacterial-modified metabolites (hippurate, 3-hydroxy-3-phenylpropionate, and cinnamoylglycine), but p-cresol-glucuronide, 3-indoxyl sulfate, indole propionate, and trimethylamine N-oxide were significantly elevated in the plasma of Mediterranean diet consumers (Figure S3). These data suggest that the elevated mammary gland phenylalanine-derived bacterial-modified metabolites may be site specific, possibly through modification by mammary gland localized microbiota, as plasma levels remained unchanged.

taxonomies and plotted the relative abundances. Consumption of Mediterranean diet was associated with significantly increased mammary gland Fusobacteria phylum populations, compared with a Western diet (Figure 2A). Consumption of Mediterranean diet was associated with decreased mammary gland abundance of Ruminococcaceae and Lachnospiraceae bacteria and increased Lactobacillaceae populations compared with a Western diet (Figure 2B). After aggregating OTU abundances into genus-level groupings (Figures 2C and 3), we observed an approximate 10-fold higher Lactobacillus abundance in the Mediterranean diet versus the Western diet group. Western diet-fed monkeys also had significantly higher abundance of Ruminococcus, Lachnospiraceae, Oscillospira, and Coprococcus genus bacteria in their mammary gland tissue.

We used an untargeted global metabolomics approach to determine metabolite difference in mammary glands regulated by diet. Mediterranean diet consumers had significantly higher mammary gland levels of the bile acid metabolites cholate, glycocholate (GCA), taurocholate (TCA), and chenodeoxycholate (CDCA) compared with monkeys fed a Western diet (Figures 4A–4C and 4E). Although detectable (Figures 4D and 4F–4H), there were no significant differences in deoxycholate (DCA), glycochenodeoxycholate (GCDCA), glycodeoxycholate, or taurodeoxycholate levels. Analysis of circulating plasma bile acid metabolites showed increased circulating cholate (Figure S1A) in blood from Mediterranean diet-fed monkeys compared with Western diet-fed animals. No other bile acid metabolites were significantly different between dietary groups (Figures S1B–S1H).

In the past decade, high levels of interest in gut microbiota effects on metabolite processing have led to the discovery of new bacterial-mediated transformation of dietary components and their impact on host health and the development of disease

Diet along with microbial-mediated metabolites may regulate oxidative stress. We investigated the oxidative stress, inflammation, antioxidant, and reactive oxygen species-scavenging metabolite data to determine whether diet affected these processes in the mammary gland tissue (Figure 6). Consumption of the Mediterranean diet led to upregulated 14-HDoHE,

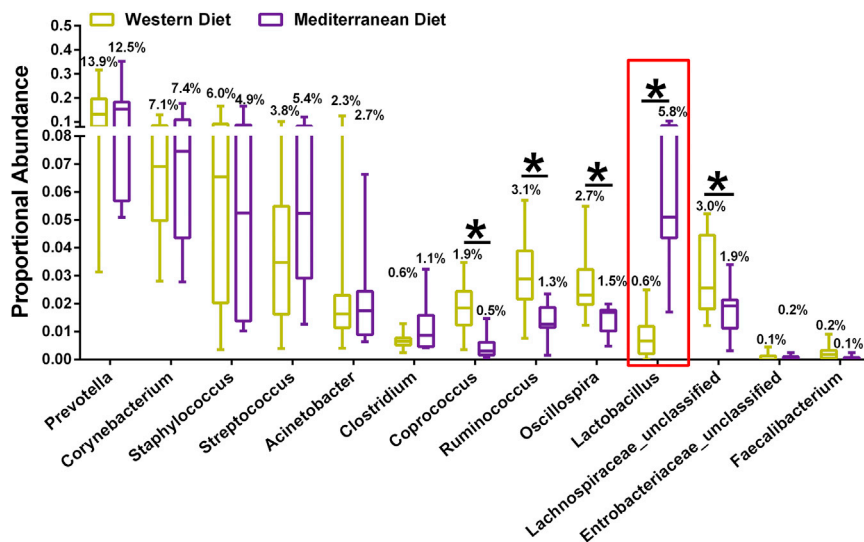


Figure 3. Differential Taxa between Western Diet-Fed and Mediterranean Diet-Fed Monkey Breast Tissue Microbiota

Taxa with nominal p values < 0.05 at the genus level are shown by * and their mean percentage proportional abundances in each diet type. Consumption of Mediterranean diet led to an approximate 10-fold upregulation of *Lactobacillus* abundance in the mammary gland. n = 11–18, *p < 0.05. Error bars show the min to max distribution.

S-methylcysteine, S-methylcysteine sulfoxide, cysteine s-sulfate, methionine sulfoxide, 5-methylribose, glutathione-reduced, and glutathione-oxidized metabolites compared with Western diet. Consumption of Western diet led to increased 13-HODE, 9-HODE, 3-hydroxystearate, and 12-HETE metabolites compared with Mediterranean diet (Figure 6A). Analysis of reduced glutathione (GSH)-to-oxidized glutathione (GSSG) ratio in the mammary gland tissues indicated elevated GSH-to-GSSG ratio in the Mediterranean diet-fed monkeys (Figure 6B).

DISCUSSION

Consumption of a Mediterranean diet was associated with distinct mammary gland microbiota populations compared with Western diets, but no differences were observed in total bacteria content or diversity (Figure 1). Similar to human studies determining effects of Mediterranean diets on gut microbiota populations, *Ruminococcus* abundance was significantly decreased in mammary gland tissue of the Mediterranean diet group, suggesting some commonly regulated microbiota population between gut and mammary gland tissues (De Filippis et al., 2016). Unlike the human gut microbiome, consumption of Mediterranean diet was not associated with non-human primate mammary gland *Prevotella* abundance and was associated with reduced *Lachnospiraceae* abundance, suggesting that there may be differential regulation of microbiota by diet depending on tissue type (Figures 2 and 3). The fecal microbiome of a subset of these monkeys consuming either a Western (n = 10) or Mediterranean (n = 10) diet was also explored (Nagpal et al., 2018). Interestingly, the effect of diet on the fecal microbiome compared with the mammary gland microbiome displayed similarities as well as divergence. Increased *Lactobacillus* and decreased *Ruminococcus* and *Coprococcus* populations were observed in both mammary gland and feces of Mediterranean diet-fed monkeys. For *Oscillospira*, there were higher levels in feces and lower levels in mammary gland of Mediterranean diet-fed monkeys compared with the Western diet-consuming

counterparts. Some microbiota populations differed by diet group in the feces but not in the mammary gland, as *Faecalibacterium* and *Prevotella* were higher in the feces but not mammary gland of Mediterranean diet-fed monkeys.

Using a non-human primate model, we demonstrated that diet alone may modulate mammary gland microbiota population. Consumption of Mediterranean diet resulted in an approximate 10-fold increase in mammary gland *Lactobacillus* abundance compared with mammary tissue from Western diet-fed monkeys. *Lactobacillus* is often thought of as commensal bacteria and is commonly used in probiotic formulations. Oral consumption of *Lactobacillus* decreased triple-negative 4T1 breast cancer tumor growth in a syngeneic breast cancer model (Aragón et al., 2014; Maroof et al., 2012). This anti-tumor effect is thought to be mediated by immune cell modulation. For example, *Lactobacillus* administration in breast tumor-bearing mice altered cytokine production in favor of an anti-tumor Th1 profile (Maroof et al., 2012). Others showed that oral *Lactobacillus* increased immune cell stimulation in intestinal Peyer’s patches with subsequent increased migration to a host of other organ locations, including mammary gland tissue, implicating *Lactobacillus* supplementation as a possible breast cancer prevention agent (Brandtzaeg et al., 2004; de Moreno de LeBlanc et al., 2005). Intestinal immune stimulation followed by migration to distant organ sites represents one possible mechanism by which oral probiotics may affect tumorigenesis. However, lactating women given a daily oral dose of *Lactobacillus* displayed elevated *Lactobacillus* populations in their breast milk, suggesting that oral consumption of probiotics can directly affect the mammary gland microbiome (Jiménez et al., 2008). Moreover, orally administered *Lactobacillus salivarius* in late pregnancy significantly decreased the rate and severity of lactation mastitis, giving further evidence in support of orally administered bacteria modulating breast health (Fernández et al., 2016). Our study now provides strong evidence that dietary intake alone may modulate the mammary gland microbiome (Figures 2C and 3), especially because subjects were living side by side in the same building, representing a breast cancer prevention model.

We found elevated bile acid metabolites (Figure 4) in mammary glands of monkeys fed a Mediterranean diet compared with those fed a Western diet. The presence of bile acid in cystic breast tissue was identified decades ago in humans (Javitt

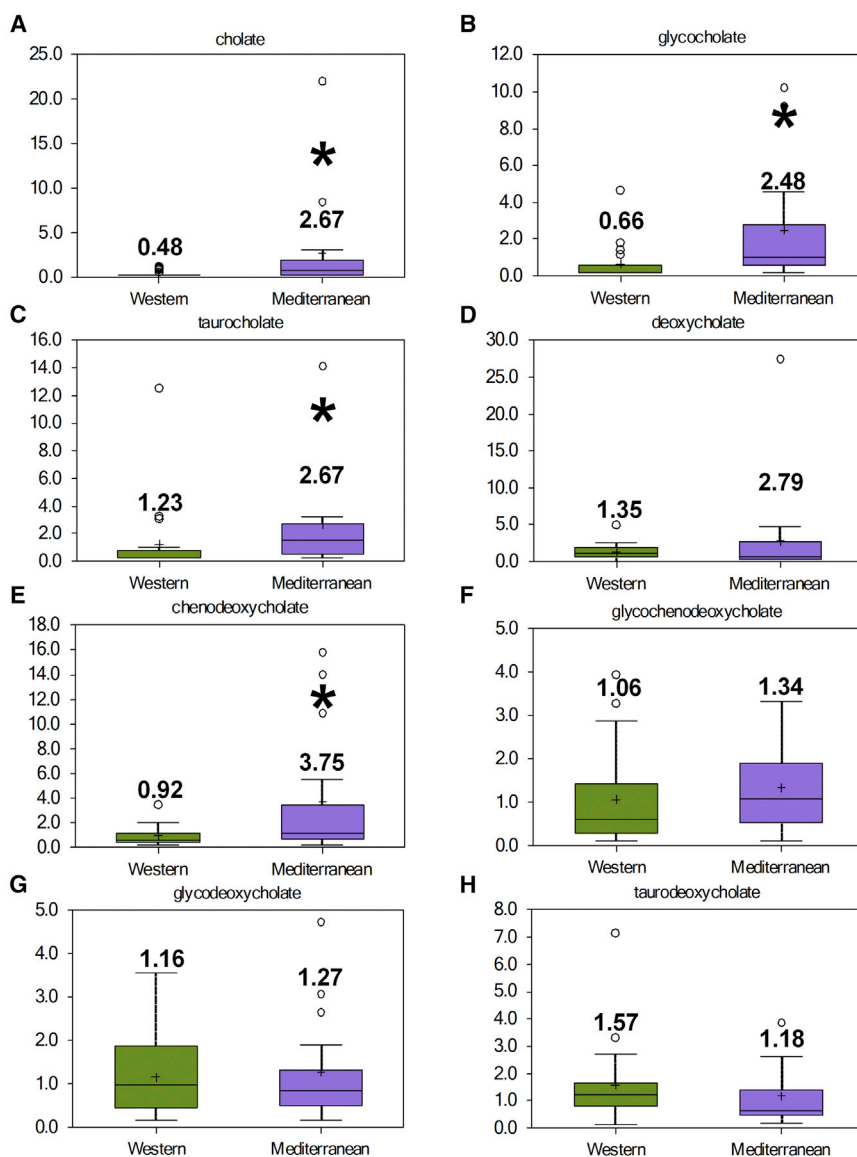


Figure 4. Diet Differentially Modulates Bile Acid Metabolite Levels in the Mammary Glands

Bile acid metabolites were determined by global untargeted metabolomics analysis of mammary gland samples from monkeys consuming a Western or Mediterranean diet for 31 months.

- (A) Cholate (CA).
 (B) Glycocholate (GCA).
 (C) Taurocholate (TCA).
 (D) Deoxycholate (DCA).
 (E) Chenodeoxycholate (CDCA).
 (F) Glycochenodeoxycholate (GCDCA).
 (G) Glycodeoxycholate (GDCA).
 (H) Taurodeoxycholate (TDCA).

n = 17–21, *p < 0.05. Error bars show the min to max distribution.

et al., 1994). In women given oral labeled CDCA before breast cyst aspiration, CDCA levels were concentrated in the cyst fluid rather than circulating in the plasma. Moreover, farnesoid X receptor, a bile acid receptor, was identified both in benign and malignant breast tissue; it was associated with increased apoptosis and repressed aromatase expression (Swales et al., 2006). Therefore, elevated bile acid metabolites may increase farnesoid X receptor (NR1H4) activation; this would then decrease localized estrogen synthesis and increase apoptosis, reducing breast neoplastic potential. Treatment of the estrogen receptor-positive breast cancer cell line MCF7 with either CDCA or GCDCA resulted in differential effects on proliferation; GCDCA increased MCF7 proliferation, while treatment with CDCA reduced proliferation (Baker et al., 1992). We observed increased CDCA metabolite levels (anti-proliferative) in mammary glands of monkeys fed a Mediterranean diet (Figures 4E and 4F), with no significant

differences in GCDCA (proliferative), possibly implicating bile acid metabolite regulation as a breast cancer prevention mechanism modulated by diet. TCA and GCA metabolites were enriched in mammary glands from Mediterranean diet-consuming monkeys compared with mammary gland tissue from Western diet-consuming monkeys (Figures 4B and 4C). These conjugated bile acids are modified by the presence of microbes (Ridlon et al., 2014, 2016a, 2016b; Swann et al., 2011). Interestingly, analysis of circulating plasma bile acid metabolite levels indicated no significant regulation of GCA, TCA, or CDCA by diet (Figure S1), most likely suggesting mammary gland-specific microbial modulation of bile acid metabolites (another possibility being the preferential accumulation of bile acids in the mammary gland tissue). *Lactobacillus* contains bile salt hydrolase activity, which was previously shown to be necessary for the production of secondary bile acid metabolites that serve as agonists for farnesoid X receptors (Li et al., 2013). The enrichment of *Lactobacillus* in the mammary glands of Mediterranean diet-fed monkeys (Figures 2C and 3) may then increase mammary gland-specific secondary bile acid metabolites to activate farnesoid X receptor (NR1H4) signaling for anticancer properties. Elevated plasma DCA was observed in postmenopausal women with newly diagnosed breast cancer compared with healthy age- and BMI-matched control plasma samples (Costarelli and Sanders, 2002a, 2002b). DCA is a mutagenic compound (Watabe and Bernstein, 1985). We observed no significant changes in DCA bile acid metabolite levels by diet, but there was a trend toward decreased DCA in the plasma of Mediterranean diet-fed monkeys (Figure S1).

On the other hand, estrogen metabolites are conjugated to bile acids for excretion. Bacteria with β -glucuronidase activity can

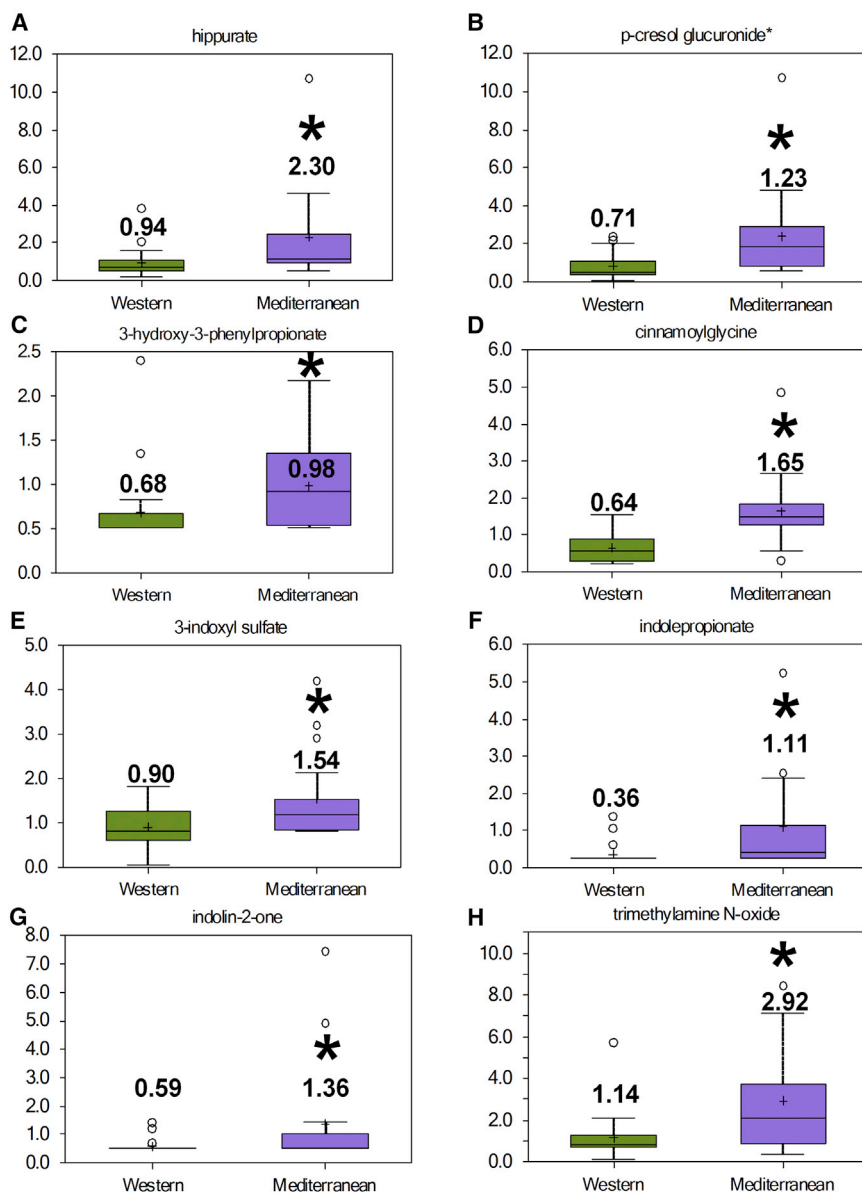


Figure 5. Diet Differentially Modulates Bacterial-Processed Bioactive Compounds

(A) Hippurate.
 (B) p-Cresol glucuronide.
 (C) 3-Hydroxy-3-phenylpropionate.
 (D) Cinnamoylglycine.
 (E) 3-Indoxyl sulfate.
 (F) Indole propionate.
 (G) Indolin-2-one.
 (H) Trimethylamine N-oxide.
 n = 17–21, *p < 0.05. Error bars show the min to max distribution.

positive bacteria populations in the gut are implicated in estrogen receptor-positive breast cancer (Kwa et al., 2016), the effect of enriched breast tissue-specific β -glucuronidase positive bacteria on bile acid deconjugation of estradiol metabolites needs further investigation.

There is a growing body of evidence demonstrating an association between phenolic and phytochemical components in Mediterranean diets and important health benefits (Visioli and Bernardini, 2011). As shown in Figure 5, monkeys consuming a Mediterranean diet had significantly higher levels of several conjugated phenolic metabolites such as p-cresol sulfate (tyrosine metabolite), hippurate, cinnamoylglycine, and 3-hydroxy-3-phenylpropionate (phenylalanine metabolites) compared with the Western diet group. Other metabolites that accumulated in mammary tissue of the Mediterranean diet-fed monkeys included indole-containing substances (tryptophan metabolites) such as indolin-2-one, 3-indole sulfate, and indole propionate. These regulated polyphenol metabolites could have come from the olive oil, fruits, and vegetables enriched

in the Mediterranean diet compared with the Western diet. There were no significant differences in tryptophan, phenylalanine, or tyrosine metabolite levels in the mammary glands (parental amino acid metabolite data shown in Figure S2), suggesting that these metabolite changes may reflect changes in microbial metabolism within the two diet regimens. Further supporting the role of microbial mediated bioactive compound generation, there were no changes in plasma metabolite levels of hippurate, cinnamoylglycine, 3-hydroxy-3-phenylpropionate, or phenylalanine (Figure S3) by diet, suggesting the microbial modulation of these metabolites may be localized to the mammary gland tissue. There were significant differences in circulating plasma metabolites p-cresol sulfate, tyrosine, tryptophan, 3-indole sulfate, and indole propionate (Figures S2 and S3), possibly indicating gut microbiome-dependent systematic effects.

deconjugate estrogen from bile acids to elevate estrogen metabolite bioavailability (Kwa et al., 2016). Lactobacillus has β -glucuronidase activity, suggesting that mammary glands from Mediterranean diet-fed monkeys with high Lactobacillus abundance (Figures 2 and 3) and higher bile acid metabolites (Figure 4) may have elevated localized estrogen bioavailability because of bacterial-mediated deconjugation independent of aromatase activity. We measured circulating serum estradiol levels at the time of necropsy in monkeys consuming Western or Mediterranean diet and observed comparable levels in the two groups (p = 0.29) (Table 1). Mammary tissue levels of estradiol were not measured and could differ depending on the tissue milieu of hormone converting enzymes and hormones that may be effected by microbiota. Tissue-level regulation of estradiol will be explored further in future studies. Although β -glucuronidase-

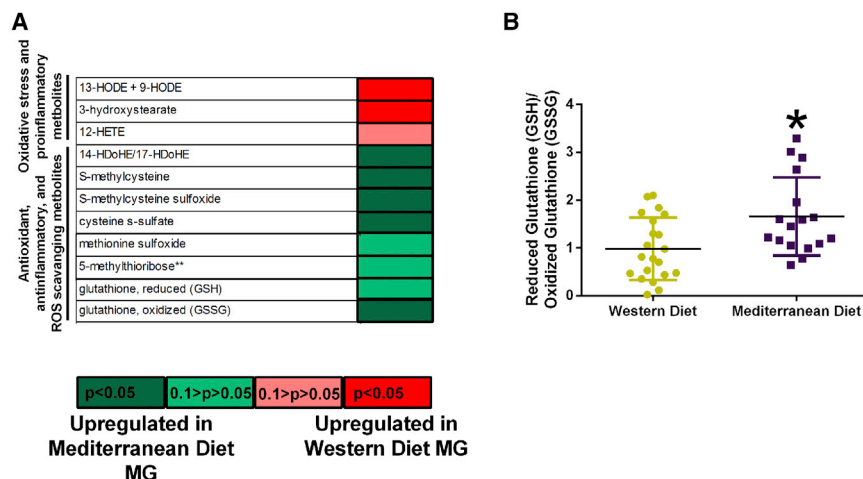


Figure 6. Diet Shifts Oxidative Stress Metabolites in the Mammary Gland Tissue

(A) Heatmap of oxidative stress, pro-inflammatory, antioxidant, anti-inflammatory, and reactive oxygen species (ROS)-scavenging metabolites in the mammary glands (MG).

(B) Reduced glutathione (GSH)-to-oxidized glutathione (GSSG) metabolite ratio in mammary glands of Western and Mediterranean diet-fed monkeys.

n = 17–21, *p < 0.05. Error bars show SD.

Elevated hippurate is a phenylalanine metabolomic marker associated with increased gut microbiome diversity and elevated fruit and vegetable consumption and negatively correlated with metabolic syndrome risk in human patients (Pallister et al., 2017). Another metabolic benzoate-derivative marker, 3-hydroxy-3-phenylpropionate, also positively correlates with gut bacterial diversity and fruit and whole grain intake (Pallister et al., 2017). Comparison of plasma metabolite profiles between conventional and germ-free mice demonstrated the required presence of bacteria for the formation of hippurate, p-cresol sulfate, cinnamoylglycine, and 3-hydroxyl-3-phenylpropionate (Wikoff et al., 2009). We observed elevated hippurate (Figure 5A) and 3-hydroxy-3-phenylpropionate (Figure 5C) in the mammary glands of Mediterranean diet-fed monkeys. These data suggest that the levels of benzoate-derived metabolites in those fed a Mediterranean diet correlated with increased fruit and vegetable intake but not mammary gland bacterial diversity (Figure 1E).

We also observed elevated bacterial-processed tryptophan-derived metabolites 3-indoxyl sulfate (Figure 5E), indole propionate (Figure 5F), and indolin-2-one (Figure 5G). Mammary gland *Lactobacillus* (significantly higher in Mediterranean diet-fed

monkeys) and *Clostridium* (trend toward higher levels in Mediterranean diet-fed monkeys) can convert tryptophan into indole-derived bioactive products (Chyan et al., 1999; Wikoff et al., 2009; Zelante et al., 2013; Zhang and Davies, 2016),

which have diverse biological functions, including activation of aryl hydrocarbon receptor-mediated IL-22 production supporting Th17 response, stimulation of pregnane X receptor (NR112) to inhibit pro-tumorigenic NF- κ B signaling, and decreased pro-inflammatory TNF- α cytokine production. Some indole products also scavenge hydroxyl radicals, supporting an antioxidant environment (Chyan et al., 1999; Venkatesh et al., 2014; Zhou et al., 2006). Activation of Th17 immune responses may initially prevent establishment of tumor formation (Lim and Savan, 2014). Moreover, inhibition of inflammation and reduction of reactive oxygen species may prevent the development of breast cancer, highlighting possible molecular mechanisms by which dietary modulated bacterial-processed bioactive metabolites may inhibit breast cancer formation. In support of this mechanism, we show that Mediterranean diet consumers had reduced oxidative stress and pro-inflammatory metabolites in the mammary gland tissue (Figure 6). This was associated with an increase in reactive oxygen species-scavenging metabolites and decreased pro-inflammatory oxidized lipid products, suggesting that regulation of microbial-mediated metabolites by Mediterranean diet consumption may affect localized mammary gland oxidative stress.

Table 1. Non-human Primate Subject Characteristics at Baseline and after 31 Months of Diet Administration

	Western Diet (n = 21)		Mediterranean Diet (n = 17)	
	Baseline	Terminal	Baseline	Terminal
Estimated age at study onset (years)	8.8 ± 0.1		8.8 ± 0.2	
Body weight (% change)	18.43 ± 4.70*		2.60 ± 3.04	
BMI (% change)	20.76 ± 4.15*		5.90 ± 2.86	
Serum estradiol (pg/mL)	87.1 ± 21.3		57.8 ± 15.5	
Body weight (kg)	3.08 ± 0.12	3.69 ± 0.25**	3.07 ± 0.17	3.25 ± 0.23
BMI (kg/m ²)	40.16 ± 1.08	48.73 ± 2.44**	40.20 ± 1.22	42.61 ± 1.80
Total plasma cholesterol (mg/dL)	130.86 ± 5.00	157.19 ± 6.35**	133.35 ± 4.42	148.65 ± 9.50
HDL cholesterol (mg/dL)	57.86 ± 3.29	72.43 ± 6.06**	58.94 ± 2.75	57.24 ± 5.23
Plasma cortisol (μg/dL)	41.68 ± 2.12	36.80 ± 1.59*	42.23 ± 1.78	31.64 ± 1.59***

*p < 0.05, Western to Mediterranean diet comparison; **p < 0.05, Western diet baseline to terminal time point comparison; ***p < 0.05, Mediterranean diet baseline to terminal time point comparison.

In conclusion, we determined that diet significantly regulated mammary gland-specific microbiome populations in a well-established non-human primate model of women's health. Mammary gland from Mediterranean diet consumers had a 10-fold higher *Lactobacillus* abundance compared with Western diet-fed monkeys. Conversely, Western diet-fed monkeys had significantly higher abundance of *Ruminococcus*, *Lachnospiraceae*, *Oscillospira*, and *Coprococcus* genus bacteria in their mammary gland tissue. Mammary gland samples from Mediterranean diet-fed monkeys had elevated bile acid metabolites and bacterial-processed bioactive compounds that may have anticancer properties. Taken together, these data highlight the impact of diet on mammary gland-specific microbiota and metabolites, which may represent a potential mechanism by which diet can affect breast inflammation and modulate breast cancer risk. Thus, changes in diet or supplementation with selected probiotics could represent a potential regimen for breast cancer prevention.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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 - Metabolomics bioinformatics and statistics
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and four tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.08.078>.

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AUTHOR CONTRIBUTIONS

C.A.S., T.C.R., S.E.A., and T.B.C. designed the *in vivo* portion of the experiment. B.U., K.Y.J.C., T.C.R., and A.S.W. performed experiments. J.A.T. supplied plasma metabolomics data. C.A.S., T.C.R., A.C., J.A.T., and K.L.C. wrote and revised the manuscript. K.L.C. created the conceptual design of post-mortem experiments. C.A.S. and K.L.C. supervised the study. All authors discussed the results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
d7-glucose, d3-leucine, d8-phenylalanine and d5-tryptophan	Cambridge Isotope Laboratories	(Evans et al., 2014)
D5-hippuric acid, d5-indole acetic acid and d9-progesterone	C/D/N Isotopes, INC	(Evans et al., 2014)
Bromophenylalanine, tridecanoic acid, alpha-ketoglutarate, ATP, malic acid, NADH and oxaloacetic acid	Sigma-Aldrich	(Evans et al., 2014)
Amitriptyline, succinic acid, pyruvic acid and NAD+	MP Biomedicals, LLC.	(Evans et al., 2014)
DL-2-fluorophenylglycine and DL-4-chlorophenylalanine	Aldrich Chemical	(Evans et al., 2014)
Critical Commercial Assays		
Estradiol (Sensitive) ELISA kit	IBL America Minneapolis, MN, USA	# IB78239
PowerMag Soil DNA Isolation kit	Mo Bio Laboratories- QIAGEN	27000-4-KF
MiSeq Reagent Kits v2	Illumina	MS-102-2003
Total cholesterol	Alfa Wasserman	http://www.alfawassermannus.com/dt-aceaxcel.asp
HDL cholesterol	Alfa Wasserman	http://www.alfawassermannus.com/dt-aceaxcel.asp
Cortisol RIA	Diasource (Louvain-la-Neuve, Belgium)	Cortisol RIA CT KIPI-28000
Deposited Data		
Metabolomics	Metabolon Raleigh, NC, USA	Uploaded in Supplemental data (Table S3)
16S sequencing OTU proportional abundance	Microbiome Insights Vancouver, Canada	Uploaded in supplemental data (Table S4)
Experimental Models: Organisms/Strains		
Cynomolgus macaques (<i>M. fascicularis</i>)	SNBL USA SRC, Alice, TX	https://snbl.com/
Oligonucleotides		
Dual-barcoded primers	IDT	Supplemental Data, Appendix D in Kozich et al., 2013
Software and Algorithms		
Greengenes (v. 13_8)	(Schloss et al., 2009)	https://www.mothur.org/wiki/Greengenes-formatted_databases
mothur software package (v. 1.39.5)	(Kozich et al., 2013)	(http://www.mothur.org/wiki/MiSeq_SOP)
Metabolon peak identification software	(Evans et al., 2014)	https://www.metabolon.com/what-we-do/our-technology

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Katherine L. Cook (klcook@wakehealth.edu)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Subjects

All procedures involving primates were conducted in accordance with institutional, state, and federal laws for the use of nonhuman primates in laboratory settings and approved by the institutional animal care and use committee. Forty adult 8.8 year old research naive female cynomolgus macaques (*M. fascicularis*) were obtained (SNBL USA SRC, Alice, TX), quarantined in single cages for one month, and moved to social groups of four animals each which lived in pens (3 m X 3 m X 3 m) with daylight exposure, on a 12/12 light/dark cycle, with monkey chow and water available *ad libitum*.

The monkeys were habituated to their social groups, characterized during a 7 month baseline phase, and then assigned to receive either Western or Mediterranean diet for 31 months using stratified randomization, balanced on pretreatment characteristics that reflect overall health including body weight (BW), body mass index (BMI), basal cortisol, and plasma high density lipoprotein cholesterol concentrations. See [Table 1](#) for baseline and terminal metabolic parameters. BMI was calculated as previously described ([Jayo et al., 1993](#)). In brief, BW and body length (suprasternal notch to pubic symphysis) were measured at the end of 7-month baseline metabolic normalization period and again after the 31 months of experimental diet administration. BMI was calculated as $BW / (\text{body length (m)})^2$. For cortisol measurements, a morning blood samples were taken within 5 min of capture and sedation with ketamine hydrochloride (10 mg/kg) and used with commercially available coated tube RIA kit from Diasource (Louvain-la-Neuve, Belgium). Total plasma cholesterol (TPC) and HDL were measured as previously described ([Sophonsritsuk et al., 2013](#)). In brief, TPC and HDL were determined in the Wake Forest Primate Center Clinical Chemistry Laboratory using ACE cholesterol and ACE HDL-C reagents and ACE ALERA autoanalyzer from Alfa Wasserman Diagnostic Technologies (West Caldwell, NJ). Estradiol was measured in serum samples obtained at necropsy using a commercially available competitive human targeted ELISA (Estradiol (Sensitive) ELISA kit # IB78239) obtained from IBL America (Minneapolis, MN, USA). In brief, the kit components had a standard range of 3.0 to 200.0 pg/mL and a reported sensitivity of < 1.4 pg/mL. In order to remove interfering substances, serum samples were extracted with ether, the extract was then dried and reconstituted with the zero standard calibrator from the kit prior to analysis. Values from human controls from outside manufacturers were within accepted ranges. Serum sample collection for estradiol measurement was not timed by the menstrual cycle.

METHOD DETAILS

Diets

Monkeys consumed either the Western or Mediterranean Diets (See [Table S1](#). Ingredients in experimental diets. Related to [Table 1](#)) which were formulated to be isocaloric with respect to protein, fat and carbohydrates, and identical in cholesterol content (~320mg / 2000 calories/day). The Western diet was formulated to be similar to that consumed by American women age 40–49 as reported by the USDA ([U.S. Department of Agriculture, Agricultural Research Service, 2014](#)). Specifically, protein and fat were derived mainly from animal sources; the diet was high in saturated fats and sodium, and low in monounsaturated (MUFA) and n-3 fatty acids ([U.S. Department of Agriculture, Agricultural Research Service, 2014](#)). The Mediterranean diet was formulated to mimic key aspects of the Mediterranean diet. Protein and fats are derived mainly from plant sources, with some lean protein from fish and dairy, and MUFAs are relatively high ([Bédard et al., 2012](#); [Kafatos et al., 2000](#)). The Mediterranean diet n-6:n-3 fatty acid ratio was similar to a traditional hunter-gatherer type diet ([Cordain et al., 2005](#)), higher in complex carbohydrates and fiber, and lower in sodium and refined sugars (see [Table S2](#). Experimental diet composition compared with human dietary patterns. Related to [Table 1](#)). Two animals were placed on a standard monkey chow due to Mediterranean diet intolerance. The data from these animals are not shown.

Breast tissue and plasma collection

Plasma was collected after 23 months of diet consumption. Breast tissue was collected after 31 months of diet consumption. Following ketamine (15 mg/kg) sedation sodium pentobarbital was administered to attain surgical anesthesia (approximately 13 mg/kg, i.v.). Tissues were collected according to the diagram shown in [Figure 1A](#) and snap frozen at -80°C or fixed in 4% paraformaldehyde. The final sample size was $n = 21$ for Western diet and $n = 17$ for Mediterranean diet.

DNA extraction, PCR, sequencing, and sequence processing

Mammary gland specimens were placed into a MoBio PowerMag Soil DNA Isolation Bead Plate. DNA was extracted following the manufacturer's protocol. Bacterial 16S sequencing, data analysis, and interpretation were performed by Microbiome Insights, Vancouver, Canada. Bacterial 16S rRNA genes were PCR-amplified with dual-barcoded primers targeting the V4 region, following the methods of ([Kozich et al., 2013](#)). Amplicons were sequenced with an Illumina MiSeq using the 250-bp paired-end kit (v.2). Sequences were denoised, taxonomically classified using Greengenes (v. 13_8) as the reference database (https://www.mothur.org/wiki/Greengenes-formatted_databases), and clustered into 97%-similarity operational taxonomic units (OTUs) with the mothur software package (v. 1.39.5) ([Schloss et al., 2009](#)), following the recommended procedure (https://www.mothur.org/wiki/MiSeq_SOP; accessed Nov 2017). The potential for contamination was addressed by co-sequencing DNA amplified from specimens and from four each of template-free controls and extraction kit reagents processed the same way as the specimens. Two positive controls, consisting of cloned SUP05 DNA, were also included (number of copies = 2×10^6). In brief, the SUP05 16S DNA sequence was taken from environmental data via. (NCBI Sequence ID: HQ673668.1). The sequence was then synthetically created using IDT gBlocks ([https://www.idtdna.com/site/order/gblockentry\[idtdna.com\]](https://www.idtdna.com/site/order/gblockentry[idtdna.com])), which was then PCR amplified and plasmid cloned using Invitrogen's TOPO cloning kit (<https://www.thermofisher.com/order/catalog/product/K457501> [[thermofisher.com](#)]). The cloned plasmid sequence was verified using our standard amplicon sequencing protocol. Operational taxonomic units were considered putative contaminants (and were removed) if their mean abundance in controls reached or exceeded 25% of their mean abundance in specimens.

Metabolomics analysis

Samples were processed and metabolomics was performed by Metabolon, Raleigh, NC. In brief, samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards (see below) were added prior to the extraction process for quality control purposes. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent.

Authentic and Recovery standards used in non-targeted global metabolomics

Authentic standards of d7-glucose, d3-leucine, d8-phenylalanine and d5-tryptophan were purchased from Cambridge Isotope Laboratories (Andover, MA). D5-hippuric acid, d5-indole acetic acid and d9-progesterone were procured from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec). Bromophenylalanine was provided by Sigma-Aldrich Co. LLC. (St. Louis, MO) and amitriptyline was from MP Biomedicals, LLC. (Aurora, OH). Recovery standards of DL-2-fluorophenylglycine and DL-4-chlorophenylalanine were from Aldrich Chemical Co. (Milwaukee, WI). Tridecanoic acid was purchased from Sigma-Aldrich (St. Louis, MO) and d6-cholesterol was from Cambridge Isotope Laboratories (Andover, MA). Standards for the HILIC dilution series of alpha-ketoglutarate, ATP, malic acid, NADH and oxaloacetic acid were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO) while succinic acid, pyruvic acid and NAD⁺ were purchased from MP Biomedicals, LLC. (Santa Ana, CA).

Ultrahigh performance liquid chromatography-tandem mass spectroscopy (UPLC-MS/MS)

All experiments used a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The scan range varied slightly between methods but covered 70-1000 m/z. Raw data were extracted, peak-identified and processed for quality control using Metabolon's hardware and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Peaks were quantified using area-under-the-curve.

QUANTIFICATION AND STATISTICAL ANALYSIS

16S statistical analysis

Alpha diversity was estimated with the Shannon index on raw OTU abundance tables after filtering out contaminants. The significance of diversity differences was tested with an ANOVA. To estimate beta diversity across samples, we excluded OTUs occurring in fewer than 10% of the samples with a count of less than three and computed Bray-Curtis indices. We visualized beta diversity, emphasizing differences across samples, using non-metric multidimensional (NMDS) ordination. No significant pen effect was observed on microbiota populations. Variation in community structure was assessed with permutational multivariate analyses of variance (PERMANOVA) with treatment group as the main fixed factor and using 4,999 permutations for significance testing. All analyses were conducted using R software. Proportional abundance of selected microbiota were graphed and multiple unpaired t tests performed (correcting for multiple comparisons using Holm-Sidak method) was used and a value of $p \leq 0.05$ was considered statistically significant.

Metabolomics bioinformatics and statistics

The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for quality control and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.1.1 Enterprise Edition, respectively. Following log transformation and imputation of missing values, if any, with the minimum observed value for each compound, Welch's two-sample t test was used to identify biochemicals that differed significantly between Western diet and Mediterranean diet experimental groups. A p value of $p \leq 0.05$ was considered statistically significant.

DATA AND SOFTWARE AVAILABILITY

The metabolomics (Table S3. Monkey mammary gland metabolite profiles. Related to Figures 4, 5, and 6) and genus level 16S sequencing OTU proportional abundance (Table S4. Monkey mammary gland genus OTU proportional abundance. Related to Figures 2 and 3) are deposited in the supplemental data section.

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Supplemental Information

Consumption of Mediterranean versus Western Diet

Leads to Distinct Mammary Gland

Microbiome Populations

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Supplemental Table S1. Ingredients in experimental diets. Related to Table 1.

Western Diet		Mediterranean Diet	
DIET #: 1523		DIET #: 1530 (1497 + Banana)	
INGREDIENT	g/ 100g	INGREDIENT	g/100 g
Casein, USP	8.50	Casein, USP	1.74
Whey Protein 895	8.50	Whey protein - 895	1.74
		Dried Egg white	2.61
		Fishmeal (Menhaden)	2.61
		Walnuts	0.87
		Black Bean flour	4.35
		Garbanzo Bean flour	1.74
		Wheat Flour (S.Biscuit all purpose)	24.35
Dextrin	26.00	Dextrin	9.66
Sucrose	18.00	Sucrose	3.48
High Fructose Corn Syrup -55	7.00	Banana	13.04
		Applesauce	3.82
		Tomato paste	1.74
Cellulose (Alphacel)	7.94	Cellulose (Alphacel)	9.48
Total Fiber (% of diet)	7.94	Total fiber (% of diet)	12.7
Lard	4.15	Olive Oil (Filippo Berio)*	6.17
Beef Tallow HHR*	4.00	Menhaden Oil (Omegapure)	0.87
Butter, lightly salted	1.25	Butter, lightly salted	0.87
Corn Oil	3.50	Corn Oil	1.04
Flaxseed oil	0.30	Flaxseed oil	0.17
Dried Egg Yolk	0.60	Dried Egg Yolk	1.48
Crystalline Cholesterol	0.04		
Complete Vitamin Mix (Teklad)	2.50	Complete Vitamin Mix (Teklad)	2.17
Mineral Mix w/o Ca, P, NaCl	5.00	Mineral Mix w/o Ca, P, NaCl	4.35
Calcium Carbonate	0.43	Calcium Carbonate	0.37
Calcium Phosphate, Monobasic	0.75	Calcium Phosphate, Monobasic	0.65
NaCl (Table Salt)	1.60	NaCl (Table Salt)	0.63
TOTAL	100	TOTAL	100

Supplemental Table S2. Experimental diet composition compared with human dietary patterns. Related to Table 1.

	Human		Nonhuman Primate*	
	Western	Mediterranean	Western	Mediterranean
	% of Calories	% of Calories	% of Calories	% of Calories
Protein	15 ¹	17 ²	16	16
Carbohydrate	51 ¹	51 ²	54	54
Fat	33 ¹	32 ²	31	31
	% of Total Fats	% of Total Fats	% of Total Fats	% of Total Fats
Saturated	33 ¹	21 ²	37	21
Monounsaturated	36 ¹	56 ²	36	57
Polyunsaturated	24 ¹	15 ²	25	20
ω6:ω3 Fatty Acids	15:1 ⁵	2.1-3:1 ⁶	14.9:1	2.9:1
Cholesterol mg/Cal	0.13 ¹	0.16 ²	0.16	0.15
Fiber g/Cal	0.01 ¹	0.03 ³	0.02 ¹	0.04 ³
Sodium mg/Cal	1.7 ^{1,4}	1.3 ^{2,3}	1.7 ^{1,4}	1.1 ^{2,3}

¹ US Dept of Agriculture 2014, women 40-49 from NHANES *What We Eat* 2011-2012 ² Bedard,A., Riverin,M., Dodin,S., Corneau,L., and Lemieux,S. (2012). Sex differences in the impact of the Mediterranean diet on cardiovascular risk profile. *Br. J. Nutr.* 108, 1428-1434. (23)

³ Kafatos,A., Verhagen,H., Moschandreas,J., Apostolaki,I., and Van Westerop,J.J. (2000). Mediterranean diet of Crete: foods and nutrient content. *J. Am. Diet. Assoc.* 100, 1487-1493. (24)

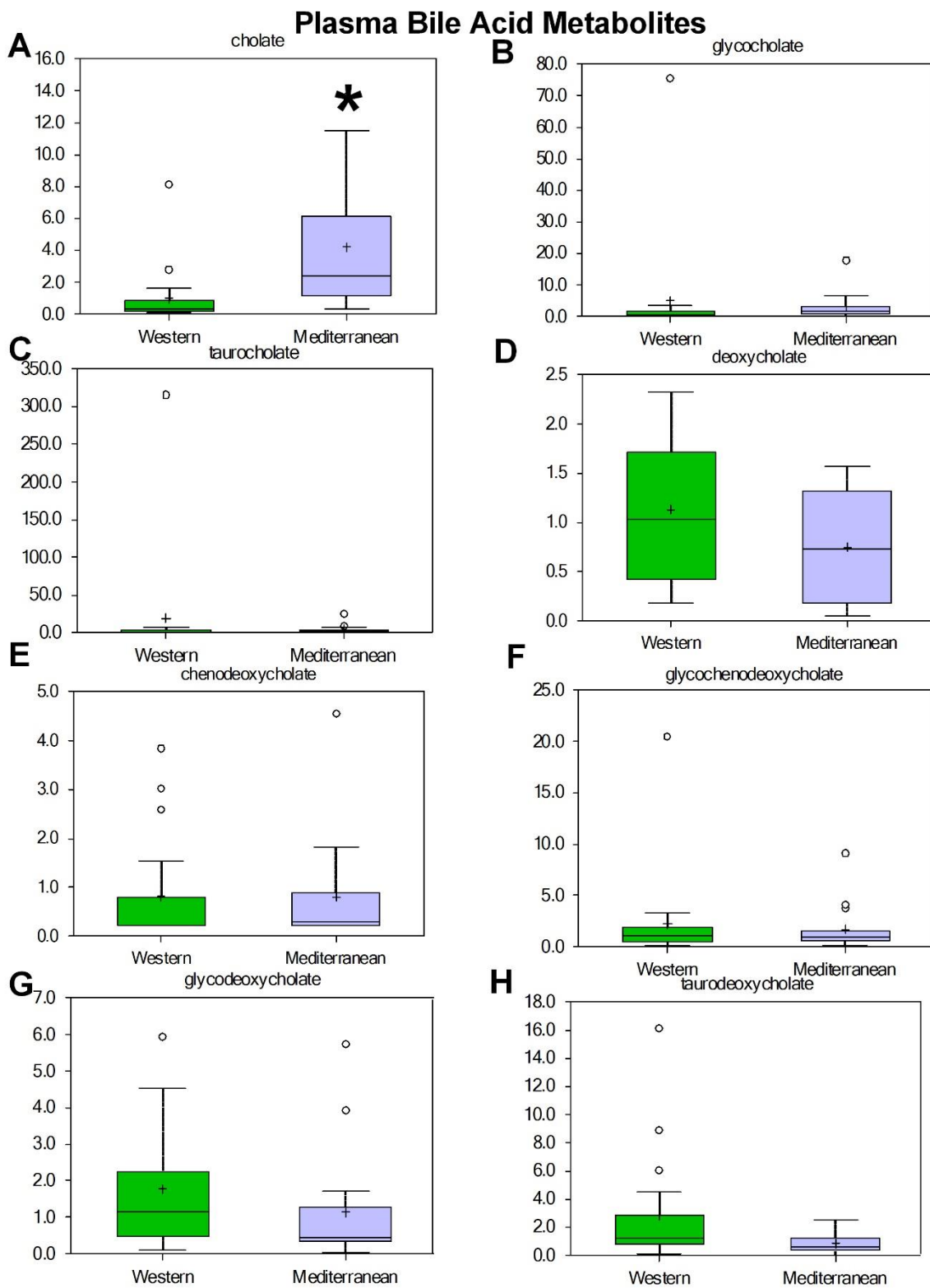
⁴ Powles,J., Fahimi,S., Micha,R., Khatibzadeh,S., Shi,P., Ezzati,M., Engell,R.E., Lim,S.S., Danaei,G., and Mozaffarian,D. (2013). Global, regional and national sodium intakes in 1990 and 2010: a systematic analysis of 24 h urinary sodium excretion and dietary surveys worldwide. *BMJ Open.* 3, e003733.(57)

⁵ Simopoulos,A.P. (2006). Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: nutritional implications for chronic diseases. *Biomed. Pharmacother.* 60, 502-507. (58)

⁶ Cordain,L., Eaton,S.B., Sebastian,A., Mann,N., Lindeberg,S., Watkins,B.A., O'Keefe,J.H., and Brand-Miller,J. (2005). Origins and evolution of the Western diet: health implications for the 21st century. *Am. J. Clin. Nutr.* 81, 341-354.(25)

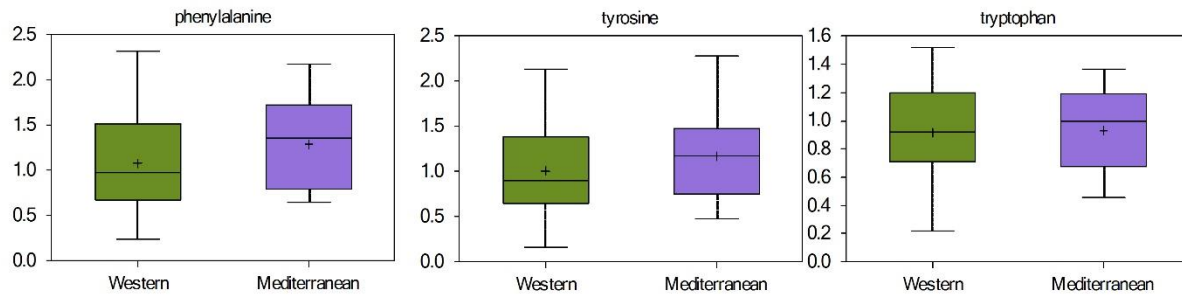
*These values were determined by diet composition analysis

Supplemental Figures and Legends:

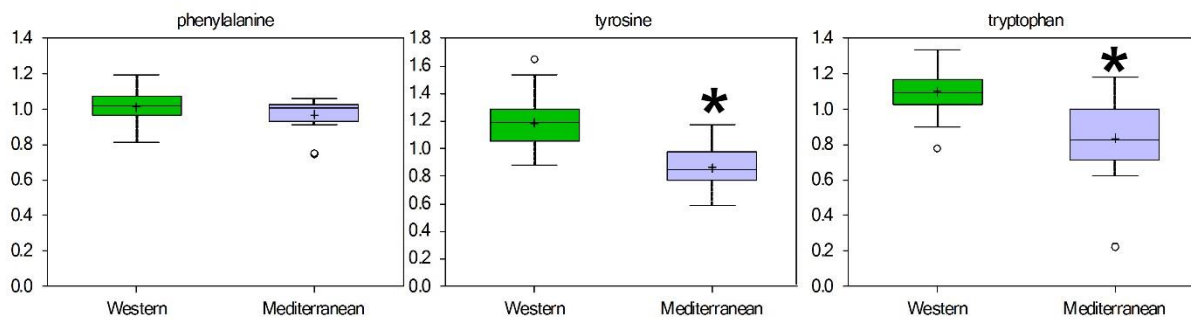


Supplemental Figure S1. Plasma bile acid metabolite levels in monkeys consuming a Western diet or Mediterranean diet for 23 months. Related to Figure 4. n=17-21; *p<0.05.

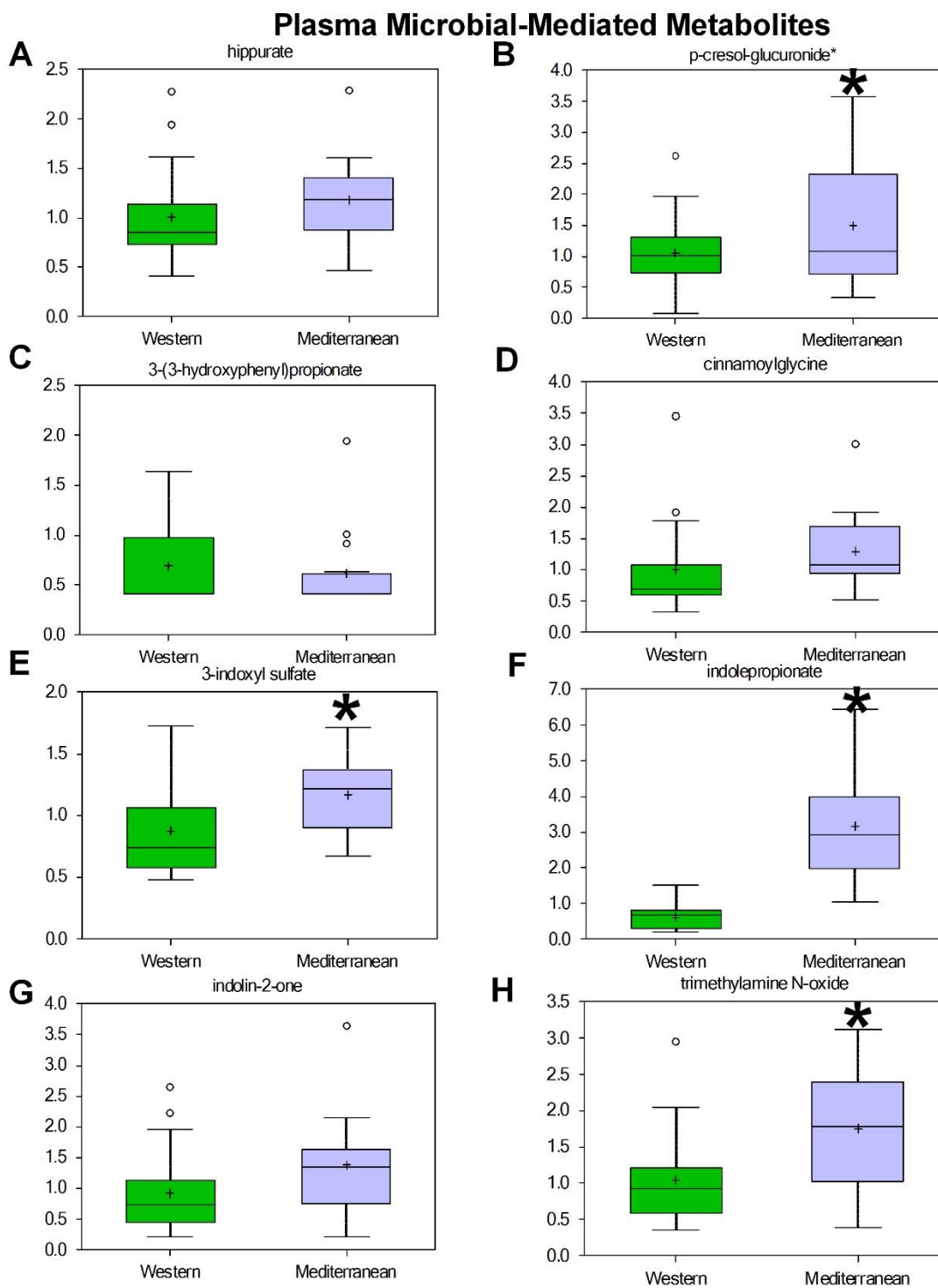
Mammary Gland Parental Amino Acid Metabolites



Circulating Plasma Parental Amino Acid Metabolites



Supplemental Figure S2. Mammary gland and plasma phenylalanine, tyrosine, and tryptophan amino acid levels in monkeys fed a Western diet or a Mediterranean diet. Related to Figure 5. n=17-21; *p<0.05.



Supplemental Figure S3. Plasma concentrations of microbial-modulated bioactive compounds in monkeys consuming a Western diet or Mediterranean diet for 23 months. Related to Figure 5. n=17-21; *p<0.05.