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Dietary fatty acids control the species of *N*-acyl-phosphatidylethanolamines synthesized by therapeutically modified bacteria in the intestinal tract.

Noura S. Dosoky, Lilu Guo, Zhongyi Chen, Andrew V. Feigley, and Sean S. Davies

Division of Clinical Pharmacology, Department of Pharmacology, Vanderbilt University, Nashville, Tennessee, USA.

Abstract

Engineering the gut microbiota to produce specific beneficial metabolites represents an important new potential strategy for treating chronic diseases. Our previous studies with bacteria engineered to produce *N*-acyl-phosphatidylethanolamines (NAPEs), the immediate precursors of the lipid satiety factors *N*-acyl-ethanolamides (NAEs), found that colonization of these bacteria inhibited development of obesity in C57BL/6J mice fed a high fat diet. Individual NAE species differ in their bioactivities. Intriguingly, colonization by our engineered bacteria resulted in increased hepatic *N*-stearoyl-ethanolamide (C18:0NAE) levels despite the apparent inability of these bacteria to biosynthesize its precursor *N*-stearoyl-phosphatidylethanolamine (C18:0NAPE) *in vitro*. We therefore sought to identify the factors that allowed C18:0NAPE biosynthesis by the engineered bacteria after colonization of the intestinal tract. We found that the species of NAPE biosynthesized by engineered bacteria depends on the species of dietary fatty acids available in the intestine, suggesting a simple method to fine tune the therapeutic effects of modified microbiota.

Keywords

N-acyl-phosphatidylethanolamines; *N*-acyl-ethanolamides; *N*-acyltransferases; fatty acids; gut microbiota; engineered bacteria

In recent years, it has become apparent that the gut microbiota may exert profound effects on host physiology including energy balance and fat accumulation. This paradigm suggests that incorporating microbes engineered to generate specific beneficial metabolites into an at-risk individual's microbiota may be useful in treating obesity associated diseases. For example,

Correspondence: Sean S. Davies, 556B RRB, Division of Clinical Pharmacology, Vanderbilt University, Nashville, TN37232-6602, USA. Phone: 615.322.5049; sean.davies@vanderbilt.edu.

Author contributions:

NSD: designed and performed most of the bacterial culture experiments and LC/MS/MS analysis, performed the pPLAAT2-EcN animal study, and assisted in writing the manuscript.

LG: designed and performed the initial bacterial experiments and LC/MS analysis, assisted in pNAPE-EcN animal studies, and assisted in editing the manuscript.

AVF: designed the PLAAT2 plasmid, assisted in initial characterization of pPLAAT2-EcN, and assisted in editing the manuscript.

ZC: assisted in initial bacterial experiments, performed the pNAPE-EcN animal studies, and assisted in data analysis.

SSD: conceived the studies, supervised the design, performance, analysis, and interpretation of the studies, and wrote the manuscript.

Conflicts of Interest: SSD, LG and ZC have a patent pending for the use of engineered bacteria expressing NAPE or NAE for treating obesity.

the enterocytes of obese individuals fail to biosynthesize sufficient amounts of *N*-acyl-ethanolamides (NAEs) (Figure 1), lipids that act as satiety factors, in response to food intake¹⁻³. We have shown that incorporating bacteria genetically engineered to biosynthesize *N*-acyl-phosphatidylethanolamines (NAPEs), the immediate precursor for NAE biosynthesis, into the intestinal microbiota has the potential to protect against development of obesity so long as there is sufficient active NAPE-hydrolyzing phospholipase D (NAPE-PLD) present in either the host or engineered bacteria to convert the NAPE to NAE^{4, 5}.

The effectiveness of such incorporated bacteria depends on their ability to sustainably produce adequate amounts of the desired metabolite (e.g. NAPE / NAEs) in the complex environment present in the intestinal tract. Unfortunately, little is known about the factors that control metabolite production by engineered bacteria once incorporated into the gut microbiota. Factors that differ in the intestinal tract versus conditions typically used for *in vitro* expression by bacteria include temperature and the availability of amino acids, fatty acids, and other components needed for biosynthesis. Biosynthesis of desired products may also be reduced if the expressed enzyme catalyzes undesired alternative products in the presence of competing substrates arising from the diet or other intestinal bacteria. Such competing substrates become particularly important if the bioactivity of their products oppose those of the desired product. In the case of bacteria engineered to produce NAPEs, the specific *N*-acyl chain incorporated into the NAPE matters because individual NAE species have differing bioactivities. For instance, *N*-arachidonyl-ethanolamide (C20:4NAE, also known as anandamide) markedly increases appetite while *N*-palmitoyl-ethanolamide (C16:0NAE) or *N*-oleoyl-ethanolamide (C18:1NAE) induce satiation and suppress appetite. So the therapeutic value of incorporating bacteria designed to increase NAPE / NAE levels depends on which species of NAE these bacteria generate *in vivo*.

We had previously found that transformation of a commensal bacteria, *E. coli* Nissle 1917 (EcN), with a plasmid for heterologous expression of NAPE synthase, the PE *N*-acyltransferase of *A. thaliana*, resulted in production of significant amounts of *N*-hexadecaenoyl-PE (C16:1NAPE), along with lesser amounts of *N*-palmitoyl-PE (C16:0NAPE), *N*-methylenehexadecanoyl-PE (C17cycNAPE) and *N*-octadecaenoyl-PE (C18:1NAPE) when these bacteria (pNAPE-EcN) were cultured in conditions typically used for bacterial expression of recombinant protein (Luria-Bertani media at 28°C)⁵. These bacteria appeared to lack the ability to produce other NAPE species such as *N*-stearoyl-PE (C18:0NAPE) or *N*-arachidonyl-PE (C20:4NAPE), even though the purified enzyme can produce these species. Administration of pNAPE-EcN in the drinking water of wild-type C57BL/6J mice significantly increased liver NAE levels and protected the mice against diet induced obesity⁵. Surprisingly, the major species of NAEs that were increased in the liver by this treatment were C16:0NAE and C18:0NAE, suggesting that once incorporated into the intestinal microbiota, pNAPE-EcN gained the ability to produce C18:0NAE and lost the ability to produce C16:1NAPE and C17cycNAPE⁵. Because individual NAPE/NAE species have differing bioactivities, it is critical to identify and appropriately control the factors that determine the species and extent of NAPE produced in the intestinal tract before attempting to utilize these engineered bacteria in human trials.

We therefore examined the effect of culture conditions and media components on the amount and species of NAPEs produced. We also examined whether different NAPE species were produced by heterologous expression of a mammalian PE *N*-acyltransferase (which uses the *sn*-1 *O*-acyl chains of phospholipids as the acyl donor^{6, 7}) in *E. coli* compared to heterologous expression of NAPE synthase, the *A. thaliana* PE *N*-acyltransferase (which uses Acyl CoA as the acyl donor⁸), that we had used previously. We found that there was a significant effect of amino acid supplementation and temperature on the species of NAPE generated; however, the most important determinant appeared to be the presence of exogenous fatty acids. Saturated and monounsaturated fatty acids significantly increased formation of their respective NAPE species, but exogenous arachidonic acid or docosahexaenoic acid had no effect. Surprisingly, the type of PE *N*-acyltransferase used for heterologous expression had relatively little effect on extent of NAPE production or the NAPE species generated. Using fecal NAPEs as a surrogate measure for NAPE biosynthesis by engineered bacteria in the intestinal lumen, we found that administration engineered *EcN* initially produced C16:1NAPE and C7cycNAPE after administration, but after colonization of mice fed high fat diet based on lard, they primarily produced C16:0NAPE, C18:0NAPE, and C18:1NAPE.

Results and Discussion

We previously found that C18:0NAE increased in the liver of mice administered *E. coli* *Nissle 1917* (*EcN*) heterologously expressing *A. thaliana* NAPE synthase (*pNAPE-EcN*) in their drinking water for 8 weeks, despite the lack of C18:0NAPE synthesis when *pNAPE-EcN* were grown in Luria-Bertani (LB) media. This result suggested that when *pNAPE-EcN* colonized the intestinal tract, it significantly altered the species of NAPE it produced. To determine if *pNAPE-EcN* did indeed change their NAPE profile after colonization, we administered *pNAPE-EcN* for two weeks in the drinking water of C57BL/6J mice fed a high-fat diet (60% kcal fat), and then measured fecal NAPE species one week after ending active administration of the engineered bacteria. We found that fecal NAPE levels were markedly higher in the mice that had received *pNAPE-EcN* compared to vehicle and that C16:0NAPE and C18:0NAPE were the predominant species (Figure 2A). No C16:1NAPE was detected in feces under these conditions. These results support the notion that the increases in hepatic C18:0NAE levels were indeed due to a shift to C18:0NAPE biosynthesis by *pNAPE-EcN* after colonization.

Because high fat diets alter several aspects of intestinal biology that might affect bacterial responses, we performed similar studies in mice fed a standard low fat chow diet. Once again, fecal NAPE levels were increased in mice that received *pNAPE-EcN*, with significant increases in C16:0NAPE, C18:0NAPE, C18:1NAPE, and C18:2NAPE, but only trivial amounts of C16:1NAPE (Figure 2B). These results confirm that *pNAPE-EcN* undergoes a significant shift in the species of NAPE biosynthesized after administration to mice, suggesting that factors present in the intestinal tract but not in our standard culture conditions altered the NAPE species biosynthesized. We therefore sought to determine how individual factors relevant to the intestinal environment affected the profile NAPE of species produced.

Rigorous determination of the factors that control NAPE biosynthesis requires completely defined media, but standard LB media contains undefined component mixtures such as yeast extract and tryptone. We therefore assessed the effect of culturing *pNAPE-EcN* in a minimal salt media (M9) supplemented with either glucose (M9g) or amino acids (M9a) as a carbon source under aerobic conditions. Control *EcN* transformed only with empty expression plasmid lacking the *A. thaliana* NAPE synthase gene (*pEcN*) grew well in all three media and, as expected, produced very little NAPE (Figure 3A). In contrast, *pNAPE-EcN* produced significant amounts of NAPE in all three of the media, with M9a media generating the highest levels of NAPE per cell (Figure 3A). In all three media, C16:1NAPE was the predominant NAPE species formed. Cultivation in M9a media resulted in a more marked difference in C16:1NAPE biosynthesis relative to other NAPE species (Figure 3B). C18:0NAPE or C20:4NAPE were not produced in any significant amount by *pNAPE-EcN* or *pEcN* in any of the three media conditions. Our finding that C16:1NAPE and C18:1NAPE levels are markedly lower in M9g compared to M9a media is consistent with previous reports that limiting available nitrogen reduces the relative abundance of C16:1 and C18:1 *O*-acyl chains in *E. coli* phospholipids⁹.

While NAPE synthase, the PE *N*-acyltransferase of *A. thaliana*, utilizes acyl CoAs as the acyl donor for NAPE synthesis, the human calcium-independent PE *N*-acyltransferase Phospholipase A/acyltransferase-2 (PLAAT2 also known as HRASLS-2), utilizes the *sn*-1 *O*-acyl chain from a second phospholipid (either phosphatidylcholine or phosphatidylethanolamine) as the acyl donor for NAPE synthesis (Figure 1). Given this difference in mechanism for NAPE synthesis, we hypothesized that heterologous expression of PLAAT2 in *EcN* would produce a NAPE profile that markedly differed from that of *pNAPE-EcN*. We therefore generated an expression plasmid for human PLAAT2 (Supplemental Figure 1), and transformed *EcN* with this expression plasmid (*pPLAAT2-EcN*). We then cultured these engineered bacteria in LB or M9a media. For comparison, we cultured *pEcN* and *pNAPE-EcN* in these same media. Surprisingly, *pPLAAT2-EcN* generated very similar NAPE profiles as *pNAPE-EcN* when cultivated in the same media (Figure 4A and B), and once again the profile produced in M9a differed from that of LB media.

To assess whether the similarity in NAPE profile for the two enzymes was characteristic of their expression in *E. coli* more generally, we also transformed the two PE *N*-acyltransferases into the common lab strains *E. coli* BSL21(*de3*) and *E. coli* DH5a and cultivated each of these bacterial strains in either LB or M9a media. While we found that individual strains of *E. coli* markedly differed in terms of their NAPE profile in each media under aerobic conditions, once again heterologous expression of human PLAAT2 gave very similar NAPE profile as heterologous expression of *A. thaliana* NAPE synthase (Figure 4C–F). Of interest, *E. coli* BSL21(*de3*) expressing either PE *N*-acyltransferase generated significantly more C17cycNAPE and C18:1NAPE than when these enzymes were expressed in *EcN*, but cultivation in M9a media still shifted the NAPE profile to significantly more C16:1NAPE synthesis than found for cultivation in LB media. Heterologous expression of the two enzymes in *E. coli* DH5a gave results intermediate between the two other *E. coli* strains, with similar C16:1NAPE synthesis but more C17cycNAPE than for *EcN*. The similarity of NAPE profile generated by the two different PE *N*-acyltransferases suggest that

their two acyl donor pools (acyl CoAs and O-acyl chains of phospholipids) are in direct equilibrium. Previous studies have shown that acyl CoA can be converted to the acyl ACP derivative for use in phospholipid synthesis^{10, 11} and that the O-acyl chains of phospholipids can undergo hydrolysis and conversion to their acyl CoA derivatives¹², providing a mechanism for this acyl donor pool equilibrium.

Culture temperature have previously been shown to alter the species of O-acyl chains present in the phospholipids of *E. coli*⁹. While the temperature within the intestinal tract of mice is approximately 37°C, we have typically prepared *pNAPE-EcN* for administration by induction at 28°C, as we had previously found this temperature gave better NAPE expression than induction at 37°C⁵. To determine the effect of temperature on NAPE expression by *pPLAAT-EcN*, we induced *pPLAAT-EcN* with and without IPTG before cultivation at 28°C and 37°C overnight in M9a media under aerobic conditions. Induction with IPTG increased NAPE biosynthesis by *pPLAAT2-EcN* 18-fold at 28°C, but only 8-fold at 37°C (Figure 5A). Furthermore, the species of NAPE significantly differed at the two temperatures, with C16:1NAPE being predominant when cultivation occurred at 28°C, but not at 37°C (Figure 5B). Levels of C16:0NAPE and C18:1NAPE were also reduced at 37°C, while levels of C17cycNAPE were increased. These findings are in keeping with previous reports that increasing cultivation temperature increases the relative abundance of saturated fatty acids while decreasing levels of monounsaturated fatty acids⁹,

LB and M9a media contain negligible amounts of fatty acids, so the only species of fatty acids available as acyl donors for NAPE biosynthesis when engineered *E. coli* are cultured in these media are those endogenously synthesized by *E. coli* themselves. The vast majority of all fatty acids in *E. coli* are esterified as the O-acyl chains of phosphatidylethanolamine and previous reports examining *E. coli* cultivated at 30°C in LB media showed that the major O-acyl chain species are C18:1 (30% total fatty acids), C16:0 (29%), C16:1 (23%), C14:0 (4.1%) and C17cyclopropane (3.4%), with no significant amounts of C18:0, C18:2, or C20:4 detected⁹. Because none of our previously tested culture conditions gave rise to C18:0NAPE biosynthesis, and both esterified and unesterified fatty acids would be present in the intestinal tract after a meal¹³, we hypothesized that utilization of exogenous fatty acids for NAPE biosynthesis accounted for the altered NAPE biosynthesis profile.

To determine if making exogenous esterified fatty acids available to the engineered bacteria could alter their profile of NAPE species, we supplemented M9a media with increasing concentrations of Tween 80, a water soluble source of esterified oleic acid (C18:1n-9 fatty acid). We found a concentration dependent increase in the extent of C18:1NAPE biosynthesis when Tween 80 was added to the culture media of *pNAPE-EcN* or *pPLAAT2-EcN* (Figure 6A). We note that our LC/MS analytical method does not distinguish between C18:1NAPE where the N-acyl chain is C18:1n-9 (which would result from incorporation of exogenously added oleic acid) from C18:1n-7 (which would result from incorporation of endogenously synthesized cis-vaccenic acid). Thus our measurement of C18:1NAPE includes both species of C18:1NAPE. C18:1NAPE represented ~15% of the total NAPE produced in the absence of Tween 80, but represented ~35% of total NAPE for Tween 80 concentration 0.25%. Absolute levels of total NAPEs produced were similar across the various concentrations of added Tween 80, so that C18:1NAPE biosynthesis appeared to

progressively displace C16:1NAPE biosynthesis as Tween 80 concentration increased in both *pNAPE-EcN* (Figure 6B) and *pPLAAT2-EcN* (Figure 6C). Above 0.25% Tween80, no further enrichment occurred suggesting that uptake of Tween80 (or oleic acid released from Tween 80) was saturated, which would prevent further enrichment of C18:1 into the acyl CoA pool and then the phospholipid *O*-acyl chain pool. Given the similarity of the results in *pPLAAT2-EcN* and *pNAPE-EcN*, we chose to focus our remaining studies on *pPLAAT2-EcN* as use of the human PE *N*-acyltransferase would be preferred over *A. thaliana* PE *N*-acyltransferase for eventual human trials of these engineered bacteria.

One initial impetus for determining the factors that controlled the species of NAPE produced by our engineered bacteria was our finding that levels of C18:0NAE were markedly increased in the liver of mice administered the engineered bacteria. We therefore sought to determine if exogenously adding stearic acid resulted in significant formation of C18:0NAPE. In the absence of added stearic acid, <1% of total NAPE was C18:0NAPE, while supplementation of M9a media with at least 0.1% stearic acid resulted in approximately 15% of total NAPE being C18:0NAPE (Figure 6D). Similar levels of total NAPEs were synthesized when M9a media was supplemented with 0 to 0.25% unesterified stearic acid, so that addition of unesterified stearic acid did not exert any toxic effects at these concentrations.

Although these results clearly indicated that exogenous stearic acid could be used for C18:0NAPE biosynthesis, the total enrichment for C18:0NAPE was less than the enrichment for C18:1NAPE that we had found with Tween80 supplementation. We hypothesized that *EcN* had a more limited capacity to take up unesterified fatty acid than fatty acids esterified in water soluble forms (i.e. Tween). We therefore determined to what extent unesterified oleic acid was utilized by *pPLAAT-EcN* when supplemented into M9a media. To our surprise, we found that supplementation of M9a media with unesterified oleic acid resulted in even greater enrichment of C18:1NAPE than did its supplementation with Tween 80 (Figure 6E). Supplementation with at least 0.025% oleic acid resulted in about 55% of total NAPE being C18:1NAPE. This result clearly demonstrated that uptake and utilization of unesterified fatty acids for NAPE synthesis was robust, but suggested that the extent of uptake and/or utilization of individual fatty acids might substantially differ.

Because oleic acid contains a double bond that stearic acid lacks, we next determined the effect of the number of double bonds present in exogenous unesterified fatty acids on their utilization for NAPE synthesis. To do this, we supplemented M9a media with a fatty acid mixture containing equimolar amounts of stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), arachidonic acid (C20:4n-6), and docosahexaenoic acid (C22:6n-3). All five of these fatty acids are typically found in human diets, although their relative abundance varies markedly. Even at the highest concentration of added fatty acids (0.5% fatty acid mixture), less than 0.5% of total NAPEs were either C20:4NAPE or C22:6NAPE (Figure 6F). In contrast, levels of both C18:1NAPE and C18:2NAPE were significantly increased by addition of the fatty acid mixture, with C18:2NAPE reaching maximal levels of 26% of total NAPE when media included at least 0.1% fatty acid mixture, and C18:1NAPE reaching 48% of total NAPE when media included 0.5% fatty acid mixture. C18:0 appeared to poorly compete with C18:1 and C18:2, as even at the highest

concentration of added fatty acids C18:0NAPE represented only 9% of total NAPEs. Our results suggest that when exogenous fatty acids are available, they are the predominant source of acyl donors for NAPE biosynthesis, but that when various exogenous fatty acids compete for utilization, the number of double bonds present is not the primary determinant of their utilization.

The inability of *pPLAAT2-EcN* to biosynthesize C20:4NAPE most likely results from the inability of *E. coli* to take up and incorporate arachidonic acid into *O*-acyl chains of its phospholipids. Exogenous fatty acid are taken up in *E. coli* by vectorial transport^{14, 15}, with the outer membrane protein FadL facilitating movement of the fatty acid through the two leaflets of the outer membrane into the acidic environment of the periplasm. Protonation of the fatty acid anion in the periplasm allows it to cross the two leaflets of the inner membrane, a process further facilitated by conversion of the fatty acid to its acyl CoA derivative by FadD. Loss of either FadL or FadD blocks uptake of exogenous long-chain fatty acids¹⁵. Previous studies found that FadL had the highest binding capacity for C16:0 fatty acid, followed by C18:1 and had poor binding capacity for C14:0 or shorter fatty acids¹⁶. FadD has been shown to utilize various saturated fatty acids from C8 to C18, with C12:0 having the highest V_{max} ^{17, 18}. To the best of our knowledge, no studies with C18:2, C20:4, or C22:6 fatty acid have been reported for either FadL or FadD. Based on our findings, we would predict that C18:2 is an effective substrate for both FadL and FadD, but that C20:4 and C22:6 fatty acids are not.

Based on these results, we hypothesized that the profile of NAPE species generated by *pPLAAT2-EcN* in the intestinal tract would undergo a change after colonization because they would be able to utilize exogenous fatty acids present in the lumen of intestinal tract from digested diets. For these studies, we fed C57BL/6J mice a high fat diet (60%kcal) based on lard that contains primarily C16:0, C18:0, and C18:1 fatty acids. We administered *pPLAAT2-EcN* at 5×10^9 cfu/ml in the drinking water of C57BL/6J mice for two weeks (experimental day 0 to day 14), and measured NAPE levels in feces collected prior to administration (experimental day -3), one week after the start of bacterial administration (experimental day 7), and one week after the end of bacterial administration (experimental day 22). As controls, fecal NAPEs were also measured in mice receiving drinking water with either vehicle only or control *EcN* without plasmid (*EcN*).

Treatment with vehicle only or control *EcN* did not significantly alter fecal NAPE levels compared to levels prior to treatment (Figure 7A). In contrast, NAPE levels were significantly higher in feces collected during bacterial administration (day 7) and after bacterial administration (day 22) from mice treated with *pPLAAT2-EcN*. In feces collected during day 7 of *pPLAAT2-EcN* administration, the predominant species of NAPEs that were increased over the controls were C16:1NAPE and C17cycNAPE, followed by C18:1NAPE and then C16:0NAPE (Figure 7B). In feces collected 8 days after cessation of *pPLAAT2-EcN* administration (day 22), the predominant species that differed from baseline were C16:0NAPE and C18:0NAPE, followed by C18:1NAPE and C18:2NAPE. These findings are consistent with the notion that in the initial administration period, the intestinal NAPE profile reflects the NAPE species generated by induction in M9a media (i.e. predominantly C16:1NAPE and C17cycNAPE), but that after colonization this profile subsequently shifts

to reflect the fatty acid composition of fatty acids in the diet fed to the animals (i.e. predominantly C16:0NAPE, C18:0NAPE, and C18:1NAPE). We note that when the matching time point and diet is compared, the fecal NAPE profile of mice treated with *pPLAAT2-EcN* (Figure 7B) is very similar to the profile of mice treated with *pNAPE-EcN* (Figure 2A). This would be expected from the results of the *in vitro* studies showing that the two strains generated similar NAPE profiles. We also note that mice treated with *pNAPE-EcN* but fed a chow diet have relatively high levels of C18:2NAPE, consistent with the utilization of the relatively high levels of C18:2 fatty acid reported for this diet.

Our finding that fecal NAPE levels from mice previously treated with *pPLAAT2-EcN* through experimental day 14 continue to be elevated at day 22 is consistent with the notion that colonized *pPLAAT2-EcN* continue to biosynthesize NAPE via PLAAT2 expression. How induction of PLAAT2 expression continues in the intestinal tract during these eight days is unclear. Basal *PLAAT2* gene expression from our plasmid is repressed by the binding of LacI to the lac operator sequence near the T7 promoter (Supplemental Figure 1), so that binding of an inducer to LacI, which leads to the release of LacI from the lac operator, is required to induce robust PLAAT2 expression and NAPE biosynthesis (Figure 4). Although we add isopropyl β -D-1-thiogalactopyranoside (IPTG), a synthetic ligand for LacI, to *pPLAAT2-EcN* cultures to induce PLAAT2 expression prior to administration, subsequent centrifugation of *pPLAAT2-EcN* followed by removal of supernatant and resuspension of *pPLAAT2-EcN* in the drinking water with 0.125% gelatin likely removes almost all of this IPTG. It is therefore unlikely that sufficient residual IPTG remains in the intestinal tract seven days after last bacterial administration (day 22) to induce PLAAT2 expression. It seems more likely that native ligands for LacI derived from the diet such as β -galactosyl glycerol or related β -galactosides^{19, 20} are the primary inducers of PLAAT2 expression in colonized *pPLAAT2-EcN*.

In summary, our studies demonstrate that the remarkable switch in the NAPE species found when engineered bacteria are administered to mice results from their utilization of exogenous dietary fatty acids. This suggests that the NAPE profile of individuals treated with *pPLAAT2-EcN* or *pNAPE-EcN* could be optimized simply by changing the fatty acid profile of their diet. Individual NAEs differ in their efficacy for various therapeutic bioactivities, so that the desired NAPE species will vary depending on the particular clinical indication that these engineered bacteria are used to treat. Our previous studies using *pNAPE-EcN* focused on inhibiting obesity by reducing food intake and increasing basal metabolic rate. In this regard, enhancing C18:1NAPE production by enriching diets with oleic acid may be beneficial, as others have reported that C18:1NAE is the most potent NAE at reducing food intake in rodents^{21, 22}. Altering the fatty acid profile of a high fat diet to enrich for C18:1n-9 (or for n-3 fatty acids) has already been utilized to reduce the adverse effects of the high fat diet on bacterial infection²³, so such enrichment with C18:1n-9 could provide dual benefits in *pPLAAT2-EcN* treated individuals. Further testing to determine the optimal NAPE profile for obesity treatment is needed; however, as one group has reported that C18:0NAE is most potent at inhibiting very short term food intake after overnight fasting²⁴. One reassuring implication of our results is that it should not be necessary to deplete diets of arachidonic acid (which is abundant in the diet of many Western countries) to achieve anti-obesity effects because *pPLAAT2-EcN* does not utilize arachidonic acid to

form C20:4NAPE, which would lead to increased C20:4NAE levels and enhanced feeding drive.

For treating clinical conditions besides obesity, enhancing levels of other NAPE species besides C18:1NAPE may be beneficial. For instance, other beneficial effects of NAEs include analgesia and reduced inflammation. In regards to treatment of chronic pain, enhancing C16:0NAPE biosynthesis may be most beneficial as a meta-analysis of data from twelve clinical studies demonstrated significant effect of C16:0NAE administration on neuroinflammation and chronic pain²⁵. Again, the optimal NAPE profile for chronic pain treatment would need to be characterized as C18:1NAE²⁶ and C18:0NAE²⁷ have also been reported to exert anti-inflammatory and analgesic effects.

Experimental Procedure

Materials:

Magnesium sulfate, triethylammonium acetate buffer, Tween 80, and methylamine solution (40 wt. % in water) were purchased from Sigma-Aldrich (St. Louis, MO). Organic solvents including methanol, chloroform, ethanol, 1-butanol and acetonitrile were all HPLC grade purchased from Thermo Fisher Scientific (Waltham, MA). Calcium chloride dihydrate, ammonium chloride, sodium phosphate dibasic anhydrous, potassium phosphate monobasic, and sodium chloride were also purchased from Thermo Fisher Scientific (Waltham, MA). Casamino acids (vitamin-free) from ACROS organics. A total of five fatty acids were used in this study: stearic acid and linoleic acid (Matreya LLC, Pleasant Gap, PA), oleic acid, arachidonic acid and docosahexadecanoic acid (Cayman chemical, Ann Arbor, MI). Ampicillin sodium salt and isopropyl-β-D-thiogalactoside (IPTG) were obtained from Research Products International (RPI; Mt. Prospect, IL). Sep-Pak silica cartridges were purchased from the Waters Corporation (Milford, MA). Kinetex 2.6μ C18 100Å column 50×2.1 mm was purchased from Phenomenex (Torrance, CA).

Bacterial strains preparation:

E. coli Nissle 1917 (EcN) was obtained as a gift from ArdeyPharm, GmbH. The pQE-80L-At expression plasmid (vector for *Arabidopsis thaliana* N-acyltransferase) and its insertion into EcN have been previously described⁵. The plasmid for human NAPE-acyltransferase HRASLS2 / PLAAT2 (*pPLAAT2*) was synthesized on our behalf by Vectorbuilder (Santa Clara, CA). This was done by insertion into *pET-6xHis* plasmid of synthesized oligonucleotides encoding for the HRASLS2 / PLAAT2 amino acid sequence using codons optimized for bacterial expression. This plasmid puts expression of *PLAAT2* under the control of a T7 promoter and Lac operator. The nucleotide sequence encoding for HRASLS2 / PLAAT2 inserted into the *pET-6xHis* plasmid was as follows:
 ATGGCGCTGGCGCGTCCGCGTCCGCGTCTGGGTGACCTGATCGAAATCTCTCGTT
 TCGGT
 TACGCGCACTGGGCGATCTACGTTGGTGACGGTTACGTTGTTACCTGGCGCCGG
 CGTCTGAAATCGCGGGTGCGGGTGCGGCGTCTGTTCTGTCTGCGCTGACCAACA
 AAGCGATCGTTAAAAAAGAAGTCTGTCTGTTGTTGCGGGTGGTGACAACCTACCG
 TGTTAACAACAACACGACGACCGTTACACCCCGCTGCCGTCTAACAAAATCGTT

AAACGTGCGGAAGAAGAACTGGTTGGTCAGGAAGTCCGCTACTCTCTGACCTCTGACA
 ACTGCGAACACTTCGTTAACCACCTGCGTTACGGTGTTCCTCGTTCTGACCAGGTT
 ACCGGTGC GGTTACCACCGTTGGTGTTCGCGCGGGTCTGCTGGCGGCGGCGTCTC
 TGGTTGGTATCCTGCTGGCGCGTTCTAAACGTGAACGTCAGTAA.

Electro-competent *EcN* cells were transformed with *pPLAAT2* by electroporation to give *pPLAAT2-EcN. BL21Gold(DE3)* and *DH5a* were transformed either with *pQE-80L1* (QIAGEN) empty vector (to give *pEcN*, *pBL21*, and *pDH5a*), *pQE-80L1-At (pNAPE-BL21*, and *pNAPE-DH5a)* or *pPLAAT2 (pPLAAT2-BL21* and *pPLAAT2-DH5a)*.

Bacterial culture:

Bacteria were grown either in a nutrient Luria-Bertani (LB) broth supplemented with 0.1 mg/mL ampicillin or in M9 minimal medium supplemented with 0.1 mg/mL ampicillin, a carbon source (0.4% casamino acids or 0.4% glucose), 0.1 mM calcium chloride and 2.0 mM magnesium sulfate to produce optimal amount of NAPes. Cultures were allowed to grow under aerobic conditions overnight at 37°C and 200 rpm (Benchmark INCU-Shaker™ 10L) then were diluted (1:50) in the same medium used for initial culture and continued to grow at 37°C and 200 rpm until they reached an optical density (OD₆₀₀) of 0.5–0.6. OD₆₀₀ was measured on Eppendorf BioSpectrometer® Basic (1 OD₆₀₀ defined as 8×10⁸ CFU/mL). Then isopropyl-β-D-thiogalactoside (IPTG, 1mM) was added to induce NAPE expression and the cultures were allowed to grow overnight under aerobic conditions at 28°C or 37°C at 200 rpm. After recording the optical density, cells were harvested by centrifugation at 5000 g and 4°C for 15 min and the pellet was resuspended in 1 mL of 5M NaCl.

Use of exogenous fatty acids for NAPE production:

Initial cultures were grown in M9 minimal medium supplemented with 0.1 mg/mL ampicillin, 0.4% casamino acids, 0.1 mM calcium chloride and 2.0 mM magnesium sulfate overnight under aerobic conditions at 37°C and 200 rpm. Free fatty acids were prepared as separate stock solutions in ethanol that were diluted by sterile M9 minimal medium (concentration of ethanol did not exceed 2% of total culture volume), in triplicates. After overnight growth, cultures were used as inoculum to test different concentrations (0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0%) of Tween 80, stearic acid (C18:0) or oleic acid (C18:1n-9). Control medium contained no fatty acid. To determine which FFA would be preferentially used, we tested different concentrations (0–0.5%) of a mixture containing equal amounts of five FFAs: stearic acid (C18:0), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), arachidonic acid (C20:4n-6) and docosahexadecanoic acid (C22:6n-3). Cultures continued to grow under aerobic conditions at 37°C and 200 rpm until they reached an optical density (A₆₀₀) of 0.5–0.6. Then IPTG (1mM) was added and the cultures were allowed to grow overnight at 28°C at 200 rpm. After measurement of the optical density, cells were harvested by centrifugation at 5000 g for 15 min and the pellet was resuspended in 1 mL of 5M NaCl.

Measurement of NAPes by LC/MS/MS:

Extraction and methylamine hydrolysis of NAPes were performed as previously described²⁸. The bacterial pellet from a 20 ml culture or 1 ml fecal homogenate were mixed

with 6 mL pre-chilled Folch solution (chloroform/methanol, 2:1 containing 0.01% BHT), followed by addition of 1 nmol C17:0 NAPE (10 μ L of 0.1mg/ml C17:0-NAPE in chloroform) and 3 mL of water. The mixture was vortexed for 5 min, incubated in ice-bath for 30 min, and centrifuged at 4,000 g for 5 min. The resulting lower (chloroform) layer was carefully collected into a new tube. The aqueous phase was re-extracted with 4 mL of chloroform. The mixture was vortexed for 2 min and centrifuged at 4,000 g for 5 min. The organic phases were combined and dried under nitrogen. The extracted lipids were reconstituted in 1 mL chloroform and loaded onto a Sep-Pak silica cartridge pre-equilibrated with 4mL methanol then 8 mL chloroform. Lines were washed by 8 mL methanol: chloroform (1:9) then NAPEs were eluted by 8 mL of Folch solution (chloroform/methanol, 2:1). The lipids were dried under nitrogen and saved at -20°C before methylamine hydrolysis. Dried lipid extracts were dissolved in 200 μ L of methylamine reagent (methylamine solution (40 wt. %)/ methanol/1-butanol (4/4/1)) and mixed well. The reaction was incubated at 53°C for 1 h and the resulting solution was used for MS analysis.

NAPE concentration was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) after methylamine hydrolysis²⁸. Samples were analyzed on a ThermoFinnigan Quantum electrospray ionization triple quadrupole mass spectrometer operating in negative ion mode, equipped with ThermoPal Surveyor autosampler. Volume of injection was 10 μ L. The mobile phase consisted of solvent A (1mM triethylammonium acetate in water), and solvent B (1mM triethylammonium acetate in acetonitrile). The lipids were chromatographed on a Kinetex C18 column (50 mm \times 2.10 mm, 2.6 μ m, 100 \AA ; Phenomenex, Torrance, CA) with a constant flow rate of 250 μ L/min. After 0.5 min hold at 1% solvent B, the solvent was gradient ramped to 99% solvent B over 4.5 minutes, held at 99% solvent B for 1.5 min and return to 1% solvent B over 0.5 min and held for 2 min before the next injection. The electrospray needle was maintained at -3300 V. The ion-transfer tube was operated at -35 V and 270°C . The tube lens voltage was set to -180 V.

For multiple reaction monitoring (MRM) mode, mass transitions with the product ion of m/z 79.1 at the specified parent ion (Table 1) at a collision energy of 50 eV were monitored. The chromatographic results were processed in Xcaliber software (ThermoFinnigan) using 13-point Gaussian smoothing. Linear regression and correlation analysis was performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA).

Animal study:

Four to six week-old male C57BL/6J mice were individually housed in the Vanderbilt University animal facility in a 12-hour light/12-hour dark cycle. They were initially fed either a chow diet (LabDiet 5001) or a refined High-Fat Diet (Research Diets D12492, with 60% kcal% fat, 5.24 kcal/g) and acclimated for 10 days. On experimental day 0, mice started receiving their assigned treatment. Depending on the study, the treatment groups received standard drinking water either with 0.125% gelatin (vehicle), with 0.125% gelatin and 5×10^9 CFU/ml EcN (EcN), with 0.125% gelatin and 5×10^9 CFU/ml *pNAPE-EcN*, or with 0.125% gelatin and 5×10^9 CFU/ml *pPLAAT2-EcN*. Drinking water with various treatments were changed 3 times per week and treatment was provided for a total of 2 weeks (through experimental day 14). After ending bacterial treatment, mice remained on the same diet

during the follow-up period. Feces were collected before, during and after bacterial treatment.

Study approval:

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of Vanderbilt University.

Statistics:

Statistical analysis was performed using GraphPad Prism 7 for Windows (GraphPad Software, San Diego, CA). A *P* value of less than 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement:

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Abbreviations:

NAPE	N-acyl-phosphatidylethanolamine
NAE	N-acyl-ethanolamide
LB	Luria-Bertani media
M9g	M9 minimal medium+ glucose
M9a	M9 minimal medium+ casaminoacids
NAPE-PLD	NAPE-hydrolyzing phospholipase D
C20:4NAE	<i>N</i> -arachidonoyl-ethanolamide
C16:0NAE	<i>N</i> -palmitoyl-ethanolamide
C18:1NAE	<i>N</i> -oleoyl-ethanolamide
C16:1NAPE	<i>N</i> -hexadecaenoyl-PE
C16:0NAPE	<i>N</i> -hexadecanoyl-PE
C17cycNAPE	<i>N</i> -methylenehexadecanoyl-PE
C18:1NAPE	<i>N</i> -octadecaenoyl-PE
C18:0NAPE	<i>N</i> -octadecanoyl-PE

C20:4NAPE	<i>N</i> -arachidonyl-PE
EcN	<i>E. coli</i> Nissle 1917
pEcN	<i>EcN</i> transformed with empty expression plasmid
pNAPE-EcN	<i>EcN</i> transformed with a plasmid for <i>A. thaliana</i> NAPE synthase
PLAAT2	phospholipase A/acyltransferase 2
pPLAAT2-EcN	<i>EcN</i> transformed with a plasmid for human <i>PLAAT2</i> /HRASLS-2
OD₆₀₀	optical density at 600nm
IPTG	isopropyl- β -D-thiogalactoside
CFU	Colony Forming Unit
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
MRM	multiple reaction monitoring

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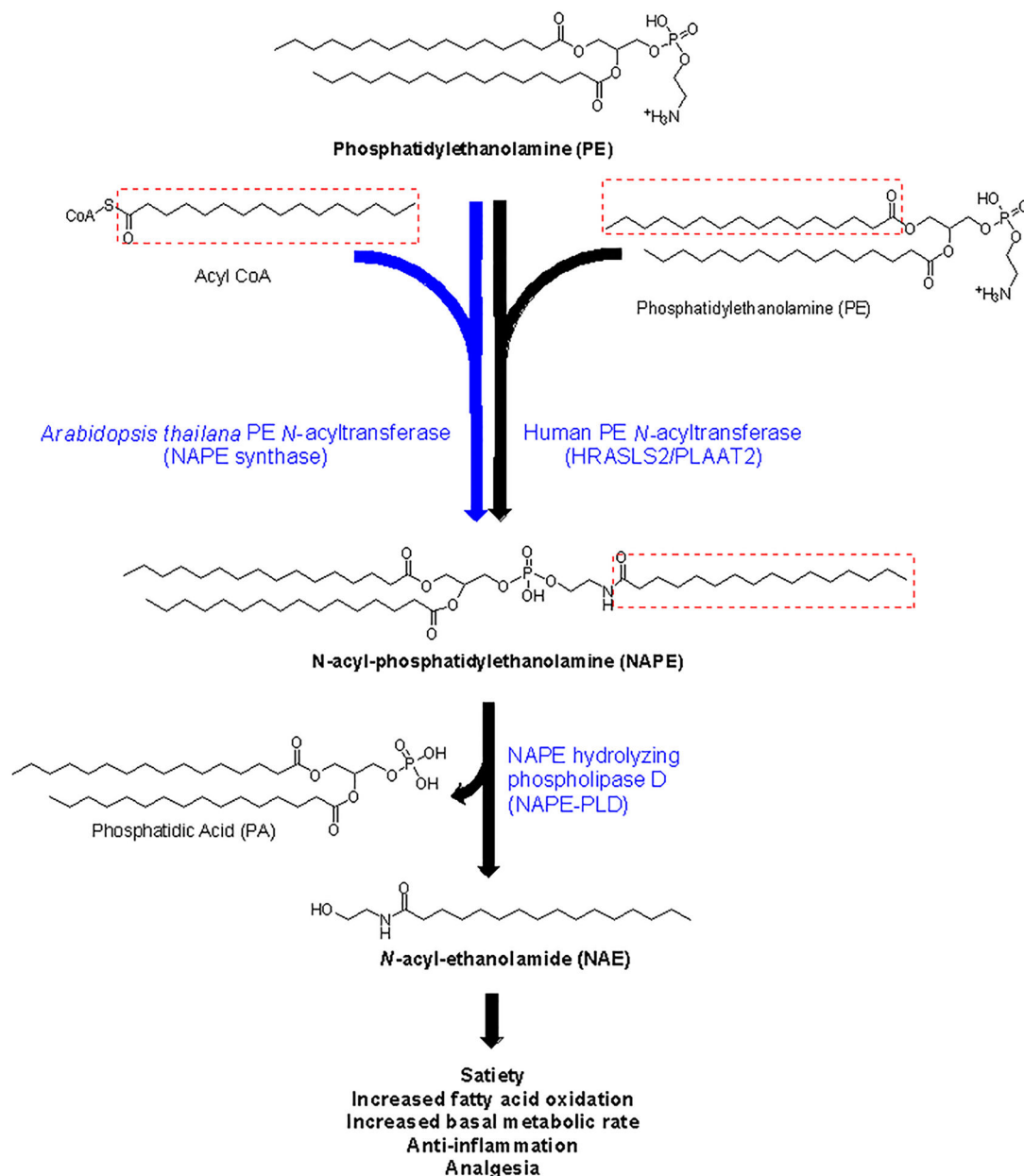


Figure 1. Biosynthesis and bioactivities of *N*-acyl-phosphatidylethanolamines (NAPEs) and *N*-acyl-ethanolamides (NAEs). *N*-palmitoyl-phosphatidylethanolamine (C16:0NAPE) is shown for illustration, but NAPE with a variety of *N*-acyl chain species can be produced by the two NAPE acyltransferases used for heterologous expression in *E. coli* in these studies. The human NAPE acyltransferase transfers an acyl group from the *sn*-1 group of phosphatidylethanolamine (PE) or phosphatidylcholine to a second PE molecule whereas the *A. thaliana* NAPE acyltransferase uses acyl CoA as the acyl donor to generate NAPEs. NAPE-PLD converts NAPEs to NAEs. Individual NAEs exert differing bioactivities depending on their *N*-acyl chain species.

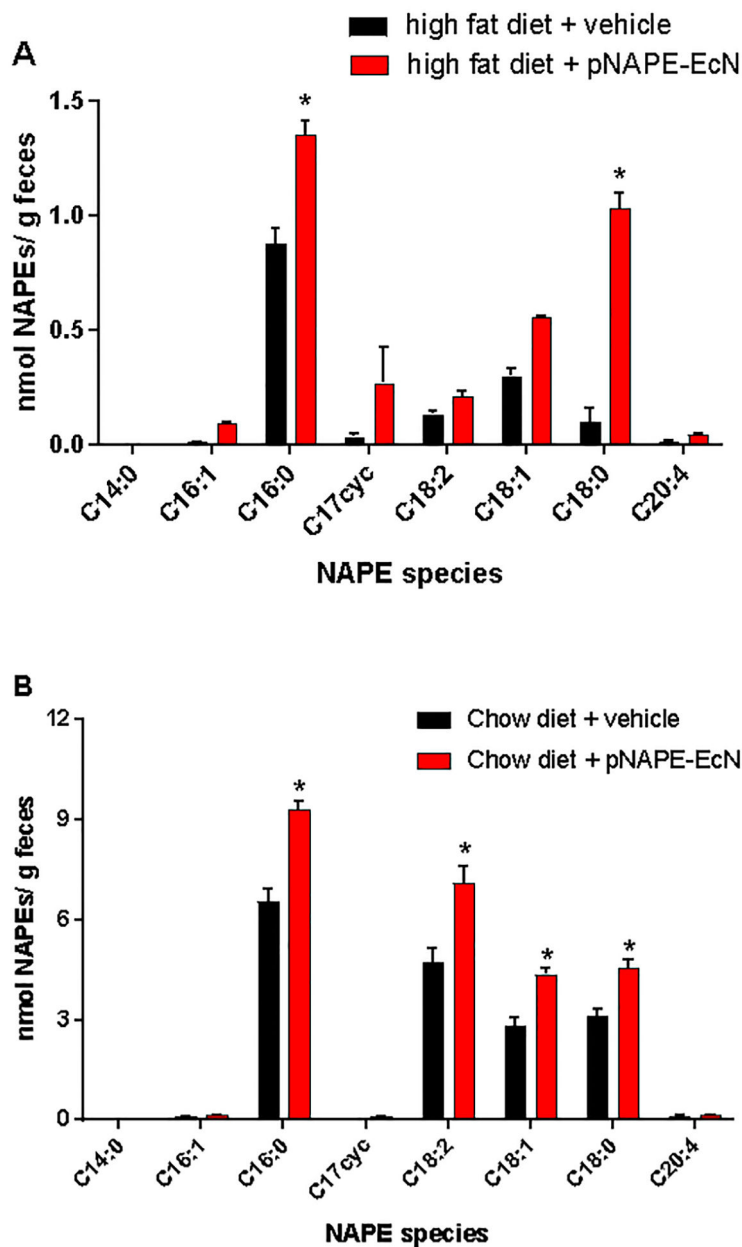


Figure 2. Administration of *pNAPE-EcN* results in increased fecal C18:0 NAPE levels. Feces were collected from mice after ending eight-week treatment with *pNAPE-EcN* or from untreated mice receiving 0.125% gelatin in standard drinking water during this same period (n=5 mice per group, mean±SEM). Fecal NAPEs were measured after extraction by LC/MS. **A.** Fecal NAPE levels of mice fed a high fat diet based on lard. **B.** Fecal NAPE levels of mice fed low fat chow diet based on plant components. For both, 2-way ANOVA $p < 0.001$ for both treatment and species. * $p < 0.05$ compared to vehicle by Sidak’s multiple comparison test.

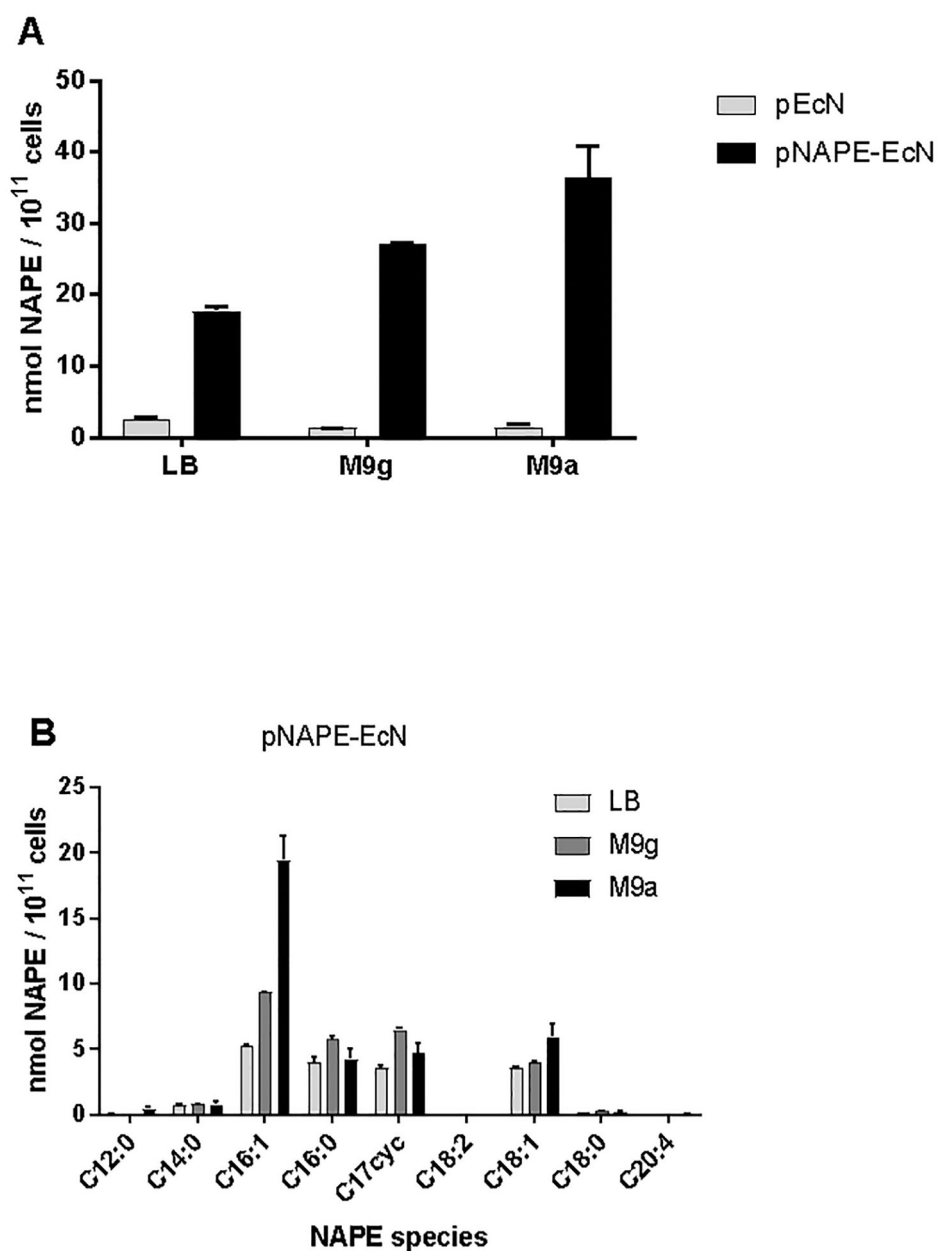


Figure 3. NAPE biosynthesis by *pEcN* or *pNAPE-EcN* in minimal media with different carbon sources. NAPEs were measured by LC/MS in bacteria grown overnight in LB, M9+glucose (M9g) or M9+aminoacids (M9a) media then induced in same media with IPTG for 18 h. **A.** Effect of media on total NAPE production by *pEcN* and *pNAPE-EcN*. **B.** Effect of media on profile of NAPE species produced by *pNAPE-EcN*.

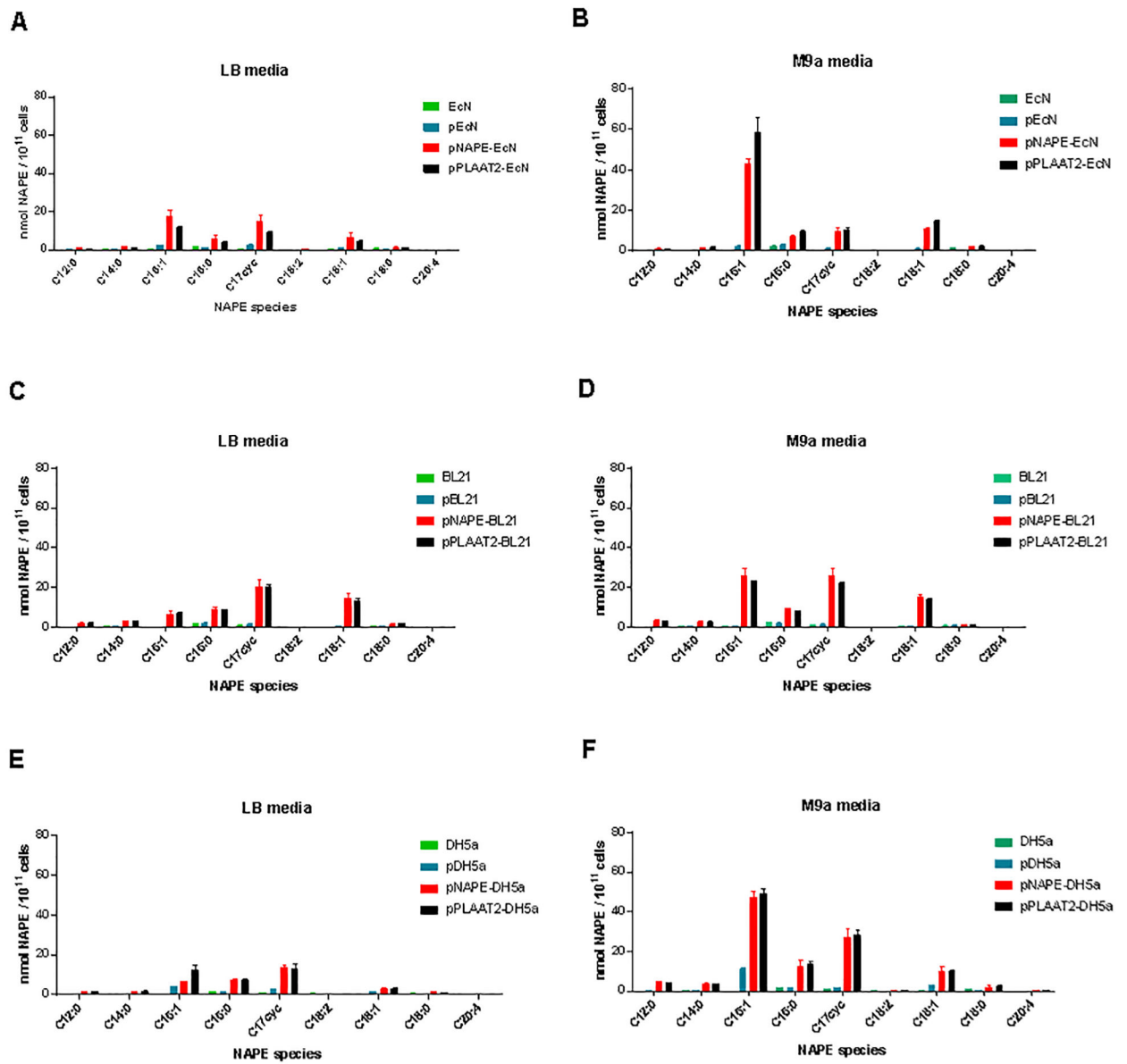


Figure 4. Comparison of NAPE profiles generated by transformation of three *E. coli* strains with human NAPE acyltransferase PLAAT2 or the *Arabidopsis thaliana* NAPE acyltransferase in minimal media. **A.** Engineered *EcN* in LB media. **B.** Engineered *EcN* in M9a media. **C.** Engineered *B21* in LB media. **D.** Engineered *B21* in M9a media. **E.** Engineered *DH5a* in LB media. **F.** Engineered *DH5a* in M9 media.

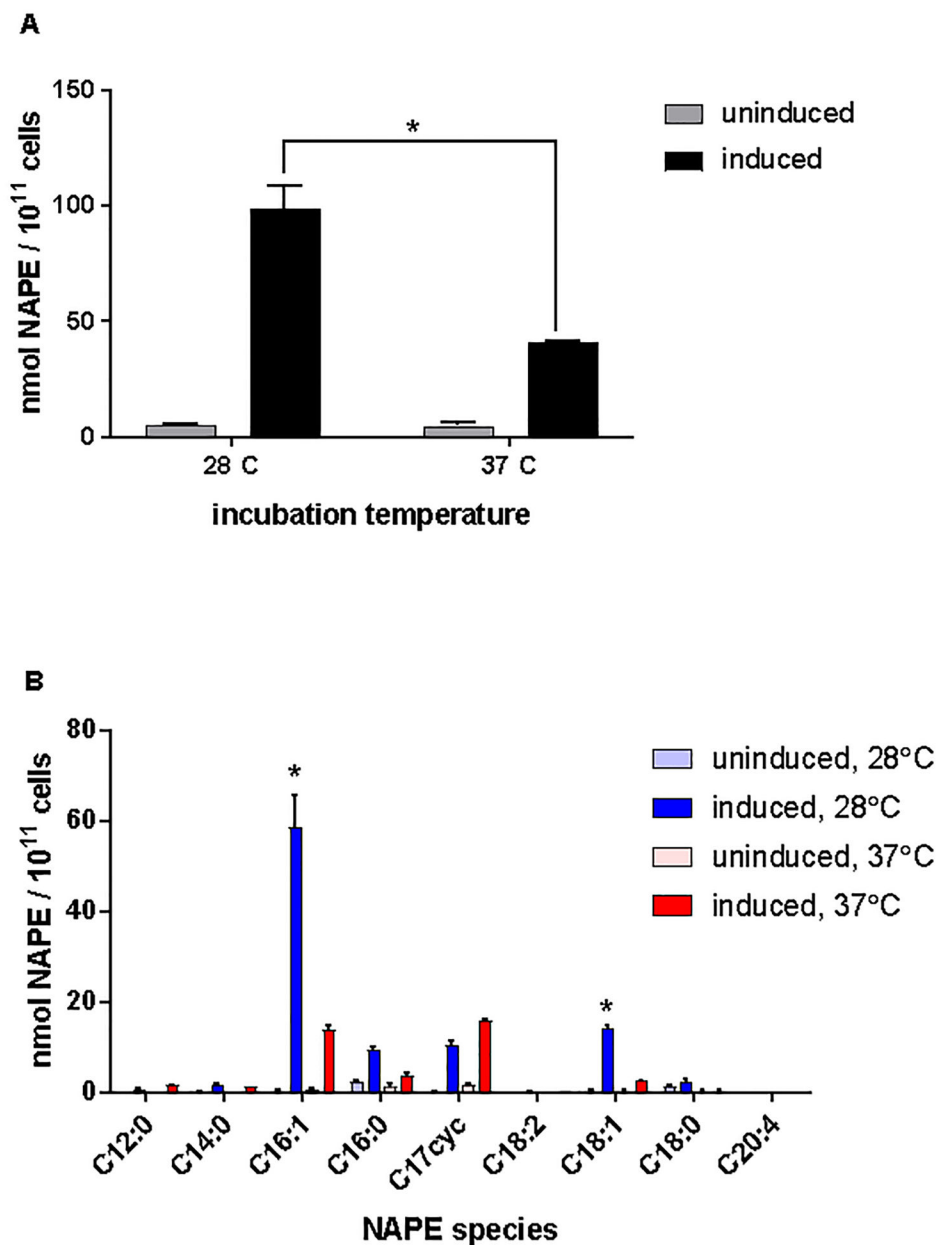


Figure 5. Effect of incubation temperature on NAPE biosynthesis by *pPLAAT2-EcN*. *pPLAAT2-EcN* were grown in M9a media, induced with IPTG and then incubated overnight at either 37°C or 28°C. NAPEs were measured by LC/MS. **A.** Total NAPEs. **B.** Profile of NAPE species produced. * $p < 0.05$ compared to 37°C.

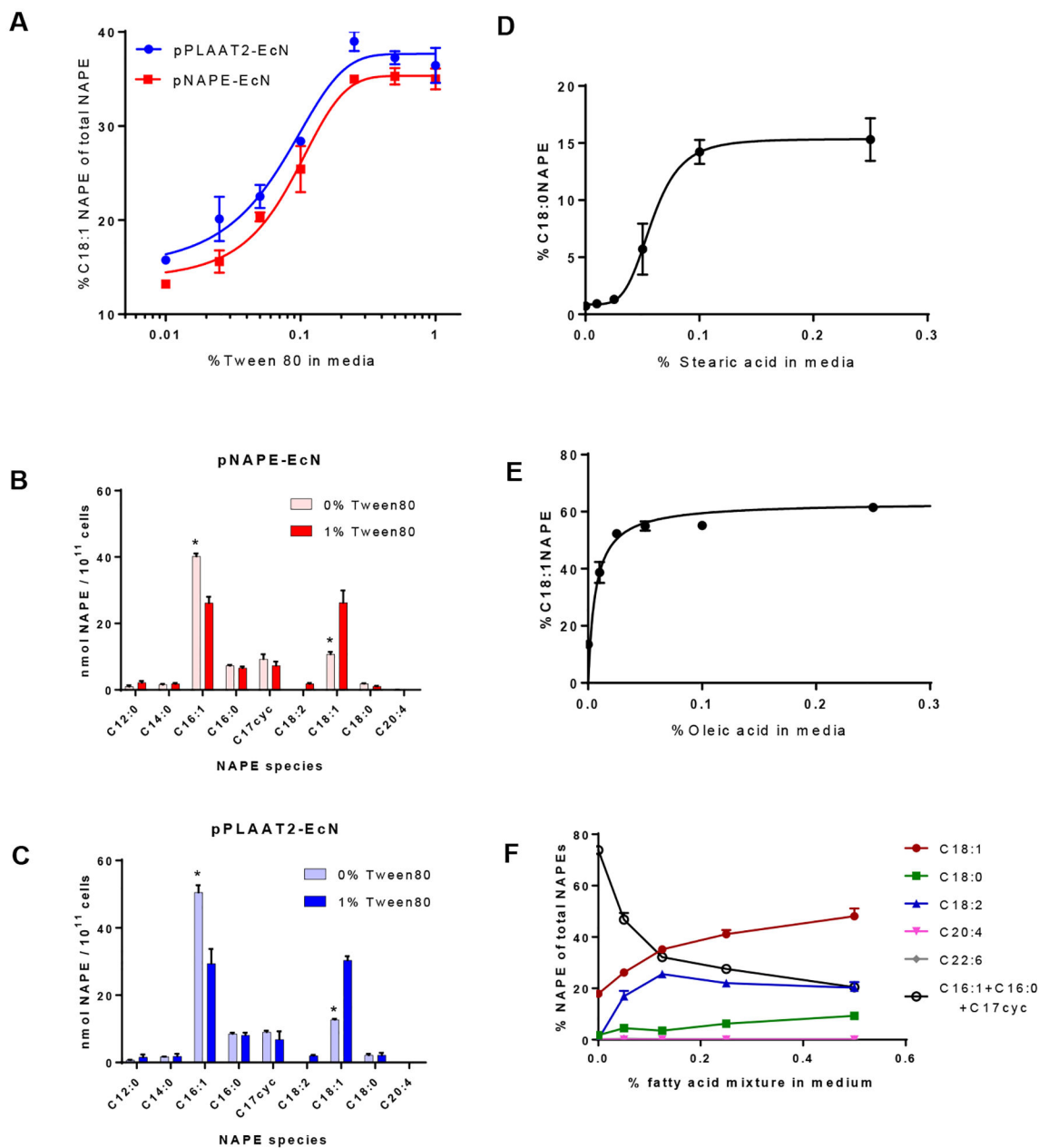


Figure 6. Exogenous fatty acids alter the species of NAPE biosynthesized by *pNAPE-EcN* and *pPLAAT2-EcN*. **A.** Effect of Tween 80 (esterified C18:1n-9) on the amount of C18:1NAPE (as % total NAPEs) biosynthesized by *pNAPE-EcN* and *pPLAAT2-EcN* grown in M9a. **B.** Effect of Tween 80 on NAPE profile for *pNAPE-EcN*. * $p < 0.05$ Sidak’s multiple comparison test for individual NAPE species. **C.** Effect of Tween 80 on NAPE profile for *pPLAAT2-EcN*. * $p < 0.05$ Sidak’s multiple comparison test for individual NAPE species. **D.** Effects of stearic acid addition on % of total NAPE biosynthesized by *pPLAAT2-EcN* that are C18:0NAPE. **E.** Effects of oleic acid addition on % of total NAPE biosynthesized by *pPLAAT2-EcN* that are C18:1NAPE. **F.** Effects of adding a mixture of five free fatty acids

(C18:0, C18:1, C18:2, C20:4, C22:6) on the profile of NAPE species biosynthesized by *pPLAAT2-EcN*. The contribution of the three most prominent NAPE species produced in the absence of the fatty acid mixture (C16:1NAPE, C16:0NAPE, and C17cycNAPE) were summed for comparison.

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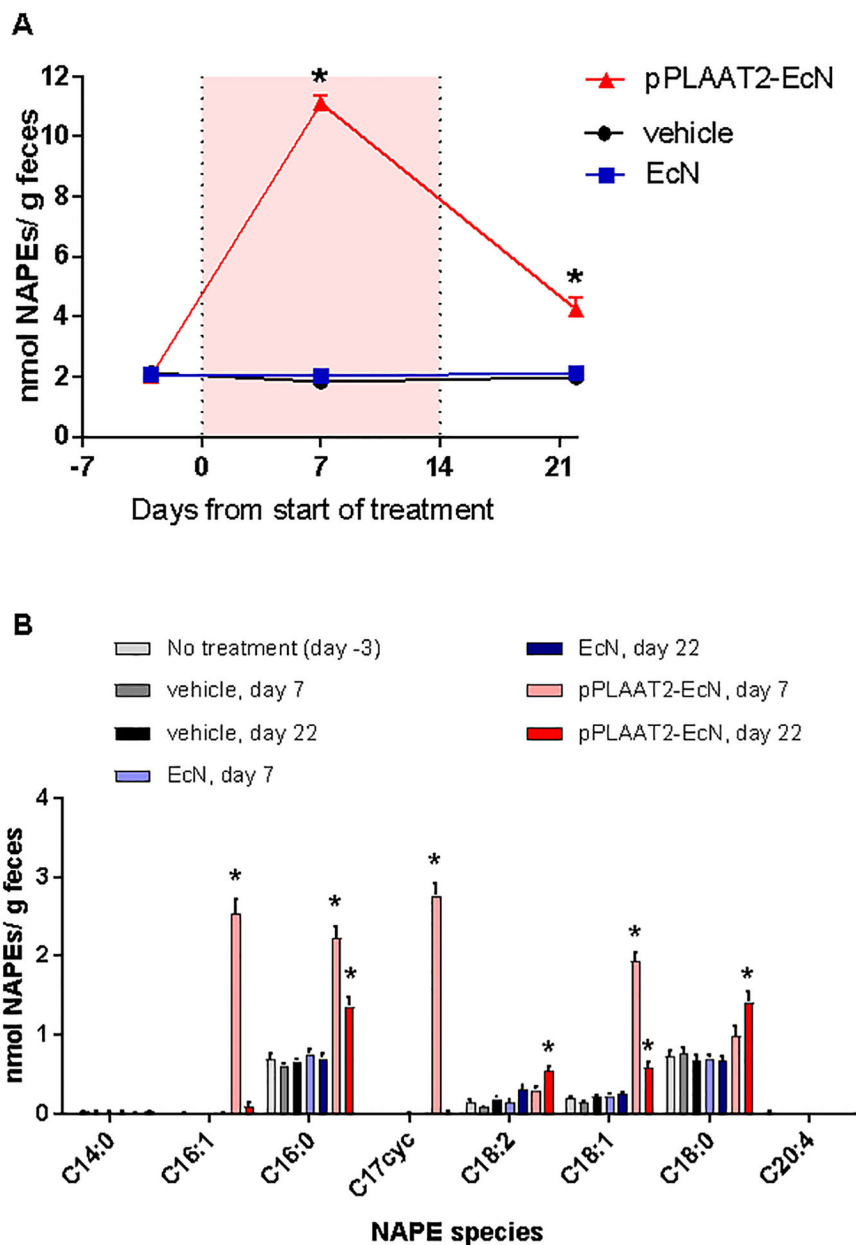


Figure 7. Effects of *pPLAAT2-EcN* administration and colonization on fecal NAPE levels and profile in mice feeding on high fat diet. **A.** Time course of total fecal NAPE levels for each treatment **B.** Effect of time and treatment on NAPE profile. 2way ANOVA $p < 0.0001$ for species and treatment. * $P < 0.05$ Dunnet post-hoc test compared to sample collected prior to treatment (no treatment).

Table (1):

Multiple reaction monitoring (MRM) parameters

Species	Precursor ion	Product ion
C12:0 GP-NAE	396.2	79.1
C14:1 GP-NAE	422.3	79.1
C14:0 GP-NAE	424.3	79.1
C15:1 GP-NAE	436.3	79.1
C15:0 GP-NAE	438.3	79.1
C16:1 GP-NAE	450.3	79.1
C16:0 GP-NAE	452.3	79.1
C17:0 GP-NAE	466.3	79.1
C17ey GP-NAE	464.3	79.1
C18:3 GP-NAE	474.3	79.1
C18:2 GP-NAE	476.3	79.1
C18:1 GP-NAE	478.3	79.1
C18:0 GP-NAE	480.3	79.1
C19ey GP-NAE	492.3	79.1
C20:4 GP-NAE	500.3	79.1
C24:6 GP-NAE	524.3	79.1