Dose-related effects of early-life intake of sn-2 palmitate, a specific positionally distributed human milk fatty acid, on the composition and metabolism of the intestinal microbiota

S. Wang,1,2* C. Zheng,3,4* D. Guo,1,2* W. Chen,1,2 Q. Xie,3,4† and Q. Zhai1,2†

1State Key Laboratory of Food Science and Resources, Jiangnan University, Wuxi, Jiangsu 214122, China
2School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China
3Heilongjiang Feihe Dairy Co. Ltd., Chaoyang, Beijing 100015, China
4PKUHSC-China Feihe Joint Research Institute of Nutrition and Healthy Lifespan Development, Haidian, Beijing 100083, China

ABSTRACT

sn-2 Palmitate in human milk plays an important role in the physiological health of infants by reducing mineral loss, improving stool hardness, and relieving constipation. Also, sn-2 palmitate modulates intestinal microbiota. However, it remains unclear whether the effects of sn-2 palmitate on infant gut microbiota are dose-dependent. In this study, we investigated the effects of low, medium, and high doses (600, 1,800, and 5,400 mg/kg body weight, respectively) of sn-2 palmitate on the structure, composition, and metabolic function of intestinal microbes in mice. Our results showed that high doses of sn-2 palmitate significantly modulated α- and β-diversity of the intestinal microbiota. The relative abundance of Lachnospiraceae_NK4A136_group decreased with increasing doses of sn-2 palmitate. In contrast, the abundances of Bacteroides phylum, Bacteroides, uncultured_Lachnospiraceae, and uncultured_Muribaculaceae were positively correlated with sn-2 palmitate doses. The number of genes predicted encoding autophagy-yeast, phospholipase D signaling pathway, and pentose and glucuronate interconversion metabolic functions of intestinal microbiota increased with increasing doses of sn-2 palmitate. In addition, low and medium doses of sn-2 palmitate significantly upregulated the arginine and proline metabolic pathways, and high doses of sn-2 palmitate significantly increased purine metabolism. Our results revealed that the effects of sn-2 palmitate intake early in life on the composition and metabolism of the intestinal microbiota of mice showed dose-related differences. The study is expected to provide a scientific basis for the development of infant formulas.

Key words: sn-2 palmitate, 1,3-dioleyl-2-palmitoylglycerol, dose-dependent effect, intestinal microbiota, metabolite

INTRODUCTION

The gut microbiota plays a crucial role in infant health. The establishment and maturation of a healthy intestinal microbiome are closely related to infant early growth and immune development (Ihekweazu and Versalovic, 2018). The intestinal microbiota promotes maturation of the immune and nervous systems, maintenance of intestinal epithelial homeostasis, resistance to invasion by pathogens, and regulation of host metabolism (Belkaid and Hand, 2014). The evolutionary process of infant intestinal microbiota is influenced by a series of external factors, such as maternal diet during pregnancy (Moossavi et al., 2019), gestational age (Korpela et al., 2018), delivery mode (Akagawa et al., 2019), feeding practices (Cai et al., 2019), and the living environment (Forbes et al., 2018). Among these factors, the feeding regimen is one of the most important and easily manipulated (Armet et al., 2022).

Human milk (HM) is the best choice for infants because breastfeeding confers protection against antimicrobial resistance, gastrointestinal infections, asthma, and overweight or obesity (Victora et al., 2016). However, breastfeeding is sometimes impossible. Infant formula (IF) is an important nutritional product for non-breastfed or partially breastfed infants. It is relevant to acknowledge differences between IF and HM in protein (Ahern et al., 2019), lactose (Ahern et al., 2019), human milk oligosaccharides (Ahern et al., 2019), lipids (Wei et al., 2019a), and others. Such differences in composition make it possible for HM and IF to exert different effects on the intestinal microbiota of infants.

Up to 98% of the lipids in HM are composed of triglycerides, formed by the esterification of 3 fatty acids at sn-1, sn-2, and sn-3 sites in the main chain (Wei et
al., 2020). Human milk fat is characterized by highest content of oleic acid (C18:1), followed by palmitic acid (C16:0) and linoleic acid (C18:2). Compared with other mammalian milks, HM has a unique fatty acid composition, of which 70% palmitic acid is esterified in the sn-2 position (Qi et al., 2018; Chen et al., 2020b). This preferred position makes it favorable for fat digestion, absorption, and metabolism (Liu et al., 2022; Zhang et al., 2022). In addition, a higher proportion of sn-2 palmitic acid in HM is associated with increased calcium absorption (Bar-Yoseph et al., 2016). In contrast, palmitic acid in milk lipids or vegetable oil is more easily hydrolyzed in the intestine by specific pancreatic lipases and released as free palmitic acid, easily bound to calcium to form insoluble calcium soaps. Calcium soaps are not absorbed by the intestine and are excreted in feces, causing loss of fatty acids and calcium, intestinal discomfort, and difficult defecation (Ghide and Yan, 2021).

sn-2 Palmitate may influence the development of infant gut microbiota. Some studies have suggested that sn-2 palmitate is involved in the regulation of the intestinal microbiota. A clinical trial showed that infants consuming IF supplemented with sn-2 palmitate and prebiotics for 4 mo had α-diversity of gut microbiota similar to that of the breastfeeding group (Zhu et al., 2021). In contrast, the relative abundance of Enhydrobacter and Akkermansia in infants consuming IF supplemented with sn-2 palmitate and prebiotics was lower than that of the breastfeeding group than that of the standard IF group (Zhu et al., 2021). Notably, prebiotics have been shown to shape the structure and function of the gut microbiota, which were also likely responsible for microbiome differences. Another clinical trial found that infants fed IF containing high sn-2 palmitate had higher Bifidobacterium counts than those fed IF containing low sn-2 palmitate after 6 wk (Yaron et al., 2013).

Most studies on sn-2 palmitate and gut microbiota have focused on gut microbiota composition, whereas metabolic functions have not been systematically investigated. Furthermore, the role of different doses of sn-2 palmitate in the composition and metabolism of the gut microbiota in early life remains unexplored. Thus, this study aimed to comprehensively analyze the effects of sn-2 palmitate on the structure and metabolites of the intestinal microbiota based on multi-omics and bioinformatics techniques and to evaluate the dose-effect correlation. The findings of this study will be useful in providing a theoretical basis for the structural and compositional optimization of IF.

MATERIALS AND METHODS

Animal Experiment Design

Three-week-old specific pathogen-free C57BL/6 male mice (n = 50) were purchased from the Zhejiang (China) Charles River Laboratory Animal Technology Co. Ltd. The mice were reared in a controlled specific pathogen-free environment (24–26°C, 40–60% humidity, 12-h light/dark cycle). After 7 d of acclimation, mice were randomly divided into 5 groups (10 mice per group). sn-2 Palmitate (1,3-dioleoyl-2-palmitoylglycerol) was purchased from Qingdao (China) Seawit Life Science Co. Ltd.

The treatment groups were as follows. Control: normal diet; milk: 0.05 g/mL nonfat milk powder solution (ratio of powder to water 1:20); low: 600 mg/kg body weight of sn-2 palmitate solution; medium: 1,800 mg/kg body weight of sn-2 palmitate solution; high: 5,400 mg/kg body weight of sn-2 palmitate solution. “Normal diet” referred to the standard formula feed, which was consumed by each group of mice. All mice had unrestricted access to drinking water. The ingredients in the normal formula feed (80% cereals, 10% animal proteins, and 10% additives) are as follows: corn, wheat shorts, wheat, and soybean meal; fish meal and chicken meal; gluten, vitamins (A, D, E, K, etc.), minerals (iron, zinc, calcium, phosphorus, etc.), amino acids (lysine, methionine, taurine, etc.), stone powder, vegetable oil, and sodium chloride. All groups were gavaged daily for 4 wk. Low, medium, and high doses of sn-2 palmitate solution were prepared from a 0.05 g/mL nonfat milk powder solution. The sn-2 palmitate dose referred to the standard of the National Food Safety Standard for the Use of Food Nutrition Fortifiers (GB 14880-2012, 2012) and the dose described by Gong et al. (2020). The body surface area conversion method was used to calculate the equivalent dose between mice and humans.

At the end of the experiment, the mice were euthanized to collect serum, feces, colonic contents, and colonic tissue. All mice received humane care, and all procedures were reviewed and approved by the Ethics Committee of Jiangnan University: JN.No20210630c0500905[264].

Determination of Serum Biochemical Index and Histopathological Analysis of Colon Sections

Serum biochemical parameters, including glucose, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total cholesterol, triglycerides, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, cholesterol, total protein, and lactic dehydrogenase,
were measured using an automated biochemical analyzer (Mindray Co. Ltd., Shenzhen, China).

The colon (approximately 0.5 cm) was fixed in 4% paraformaldehyde solution and sent to Wuhan Xavier Biotechnology Co. Ltd. (Wuhan, China) to prepare hematoxylin-eosin stained sections. Histopathological changes in the samples were observed using a digital slice scanner (3DHISTECH Ltd., Budapest, Hungary).

Analysis of Fecal Microbiota

Fecal microbiota analysis was performed as previously described (Yang et al., 2021). The total bacterial DNA in the feces was extracted according to the instructions of the FastDNA Spin Kit for Feces (MP Biomedicals). The PCR procedure was performed using primers 341 F (5′-CCTAYGGGRBGCASCAG-3′) and 806 R (5′-GGACTACNNGGATCTAAT-3′). Each PCR reaction (50 µL) contained 25 µL Taq Master Mix (2×), 1 µL genomic DNA, 1 µL 341 F (20 µM), 1 µL 806 R (20 µM), and 22 µL double-distilled water. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s, and a final extension of 72°C for 7 min. The amplified products were separated by agarose gel electrophoresis; the gel of the corresponding fragment was collected, and the DNA band was recovered using a gel extraction kit (Qiagen, Germany). The DNA concentration of the samples was measured on a Qubit 3 fluorometer (Invitrogen), and a library was constructed by mixing samples of equal mass. The fecal microbiome compositions of mice were established by amplicon sequencing of a ~500-bp fragment of 16S rRNA gene V3–V4 region through Illumina MiSeq PE300 sequencing platform using the 600-cycle MiSeq Reagent Kit v3 (Illumina). The sequences reads were processed using the QIIME 2 package (Quantitative Insights into Microbial Ecology, Flagstaff, AZ). The operational taxonomic units (OTU) were established de novo using UCLUST (Edgar, 2010) with 97% sequence identity cut off. The OTU of the V3–V4 region were assigned by the Ribosomal Database Project Naive Bayes Classifier.

Metrics of α- and β-diversity were calculated using QIIME2 and computed using the diversity plugin in QIIME2. The α-diversity metrics investigated included evenness vector, Faith’s phylogenetic diversity, observed OTU, and Shannon index. For β-diversity, principal coordinate analysis (PCoA) of Bray-Curtis dissimilarity indices was carried out, and the differences in community structure were visualized with a 2-dimensional PCoA plot. Functional profiles of the intestinal microbiota were predicted using PICRUSt2 (https://github.com/picrust/picrust2) based on the OTU tables and annotated by the Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg) according to predefined pathways.

Analysis of Short-Chain Fatty Acid Levels and Metabolomics of Intestinal Contents

Short-chain fatty acids (SCFA) in the feces of mice were analyzed using GC-MS. The specific methods have been described in prior studies (Lu et al., 2021; Wang et al., 2021). Briefly, the feces (3 pieces per mouse) were lyophilized, and dry weights of the fecal samples were recorded. Feces were mixed with 500 µL of saturated NaCl solution (Sinopharm) for 30 min. Then, the samples were acidified with 40 µL of 10% sulfuric acid (Sinopharm), and 1 mL of ether (Sinopharm) was added for SCFA extraction. The mixture was homogenized for 30 s and then centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was added to 0.25 g of anhydrous sodium sulfate to remove water. This supernatant was allowed to stand for 15 min and centrifuged again under the same conditions. Then, the supernatant was transferred into the liner of the gas-phase vial for GC-MS analysis. The injection temperature was 240°C; 1 µL of prepared sample was injected and separated on a Rtx-Wax capillary column (30 m × 0.25 mm internal diameter × 0.25 µm; Shimadzu) with helium as the carrier gas (flow rate: 2 mL/min, split ratio: 10:1). The temperature program was as follows: a temperature ramp from 100 to 140°C at the rate of 7.5°C/min and increased to 200°C by 60°C/min, and then the temperature was maintained for 3 min. The ionization temperature was 220°C. The standards of acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid (Sigma-Aldrich) were mixed and used at different concentrations and measured with the same conditions. The peak of each sample was compared with the same standard peak to obtain the concentration of each of the SCFA. Concentrations of SCFA were expressed per gram of dry matter to correct for the potential effects of feces consistency. An untargeted metabolomic analysis of the colonic contents was performed. The details of pre-processing and LC-MS analysis were obtained from our laboratory’s previous studies (Qi et al., 2022). The original data after LC-MS were loaded into Compound Discoverer 3.0 (Thermo Fisher Scientific), and relevant programs were run to obtain visual results. The binding peak type, retention time, peak area, and other conditions of metabolites compared in the positive and negative ion modes were preliminarily filtered. After obtaining the peak area data of metabolites in positive and negative ion modes, the 2 data were integrated and normalized. Then, pairwise comparisons were conducted between
the sn-2 palmitate group at different doses and in the milk powder control group. The significance between pairs was calculated using the \( t \)-test (false discovery rate <0.05). In combination with fold change (FC) <2 or >0.5 and \( P < 0.05 \), different metabolites were screened for comparison between groups. Finally, metabolic pathway analysis was performed using the MetaboAnalyst online platform (https://www.metaboanalyst.ca/).

**Correlation Analysis of Microbiome and Metabolomics**

The correlations between intestinal microbiota and metabolite data sets were calculated using R (version 4.0.3; R Foundation for Statistical Computing), and correlation heatmaps were generated using the Microeco bioinformatics cloud platform (https://bioincloud.tech/).

**Statistical Analysis**

Data were analyzed using IBM SPSS Statistics 22 and GraphPad Prism 8.0 (GraphPad Software Inc.), and the results are expressed as mean ± standard deviation. For multiple comparisons of \( \alpha \)-diversity of intestinal microbiota, differences were tested using the Kruskal-Wallis test, followed by Dunn’s multiple comparisons test. Between-group \( \beta \)-diversity of intestinal microbiota was compared using the PERMANOVA test. In other cases without special annotation, differences between groups were analyzed using one-way ANOVA followed by Tukey’s multiple comparisons test and \( t \)-test adjusted by false discovery rate. We considered \( P < 0.05 \) a significant difference.

**RESULTS**

**Effects of sn-2 Palmitate on Physiological and Serum Biochemical Indices**

Figure 1a shows the overall animal experiment scheme. The effects of different doses of sn-2 palmitate solution on body weight, serum biochemical indices, and colon tissues of the mice were analyzed. Compared with the blank and solvent control groups, different doses of sn-2 palmitate exhibited no obvious effect on body weight (Figure 1b). We detected no significant difference in weekly feed conversion ratio among groups (Supplemental Figure S1: https://doi.org/10.6084/m9.figshare.23244389, Wang, 2023). The measured values of serum biochemical indices of all mice were within the normal range (Figure 1c). The morphology of colon tissues was observed after hematoxylin-eosin staining. From the inside out (Figure 1d), the colon tissue consisted of mucous membrane layer (including epidermis layer, lamina propria, muscular layer of mucous membrane), mucous membrane substrate layer, muscular layer (inner and outer), and chorion layer. Normal histological morphology, complete epithelial tissue, and mucosal structure were observed in the colon tissues of the mice in each group without signs of inflammation, degeneration, or necrosis (Figure 1d).

**Effects of sn-2 Palmitate on Gut Microbiota Diversity, Structural Composition, and Function**

We analyzed the intestinal microbiota of the feces using 16S rRNA gene sequencing. Illumina MiSeq 16S rRNA gene sequencing of the V3–V4 region generated 1.87 million reads after quality filtering, with a mean sample depth of 39,087 ± 12,390 reads. First, the effects of different doses of sn-2 palmitate on the diversity of intestinal microbiota were analyzed. As shown in Figure 2a, the dose of sn-2 palmitate affected the \( \alpha \)-diversity of intestinal microbiota in mice. Compared with the medium- and low-dose groups, the diversity (evenness vector index and Shannon index) of the sn-2 palmitate high-dose group was significantly higher, indicating the highest uniformity and richness. These findings were consistent with the results reported by Hou et al. (2019). Figure 2b shows the \( \beta \)-diversity of the 5 groups by PCoA based on Bray-Curtis distance. The result revealed significant differences in the microbiota composition between the different groups in a PERMANOVA test (\( F = 2.45, P = 0.001, R^2 = 0.19 \)). There was much overlap in the confidence circles of medium- and low-dose groups (\( P = 0.664 \)), indicating that the 2 groups had similar intestinal community structures. The high-dose group was the most distant from the other 4 groups, with the greatest effect on \( \beta \)-diversity (vs. control, \( P = 0.001 \); vs. milk powder control, \( P = 0.018 \); vs. medium dose, \( P = 0.026 \); vs. low dose, \( P = 0.023 \)).

We further visualized the composition of the intestinal microbiota to observe the specific effects on the intestinal microbial community. The dominant phyla in each group were *Firmicutes* and *Bacteroidetes*, with the sum of abundance accounting for more than 90% of the total members of the intestinal microbiota (Figure 2c). *Deferribacteres*, *Proteobacteria*, and *Actinobacteria* followed. The relative abundance of phylum *Firmicutes* decreased with increasing sn-2 palmitate dose, whereas that of phylum *Bacteroidetes* was positively correlated with sn-2 palmitate dose (\( P = 0.016 \), Figure 3b). At the genus level (Figure 2d), the intestinal microbiota of the mice mainly consisted of *Lachnospiraceae_NK4A136_group*, *uncultured_Muribaculaceae*, *Alistipes*, *Ruminococcaceae_UCG014*, *Lactobacillus*, *uncultured_Lachno-
Figure 1. Effects of sn-2 palmitate on physiological and serum biochemical indices. Control: blank control, water; Milk: solvent control, 0.05 g/mL nonfat milk powder solution; Low: 600 mg/kg BW of sn-2 palmitate solution; Medium: 1,800 mg/kg BW of sn-2 palmitate solution; High: 5,400 mg/kg BW of sn-2 palmitate solution. (a) The animal experiment scheme. (b) Body weight of mice during the experimental period (4 wk). One-way ANOVA followed by Tukey’s multiple comparisons test. (c) Serum biochemical indices of mice in different treatment groups. One-way ANOVA followed by Tukey’s multiple comparisons test. Values are presented as mean ± SD. Significant differences (P < 0.05) among different treatments are indicated with different letters (a, b). Glu = glucose; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; TC = total cholesterol; TG = triglyceride; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; GGT = γ-glutamyl transeptidase; CHE = cholinesterase; TP = total protein; and LDH = lactic dehydrogenase. (d) Histopathological observation of colon tissues in different treatment groups. All histological sections were stained with hematoxylin-eosin. Scale bar = 50 μm.
Figure 2. Effects of sn-2 palmitate on intestinal microbiota diversity, structural composition, and functions. Control: blank control, water; Milk: solvent control, 0.05 g/mL non-fat milk powder solution; Low: 600 mg/kg BW of sn-2 palmitate solution; Medium: 1,800 mg/kg BW of sn-2 palmitate solution; High: 5,400 mg/kg BW of sn-2 palmitate solution. (a) α-Diversity of intestinal microbiota in different treatment groups. For violin plots, outer shading indicates the kernel density estimation of the data, upper and lower edges of boxes indicate the interquartile range, midlines indicate the median, lower and upper whiskers indicate the 95% CI, and dots indicate the outliers. Kruskal-Wallis test followed by Dunn’s multiple comparisons test. Faith_pD = Faith’s phylogenetic diversity; OTU = operational taxonomic units. (b) β-Diversity of intestinal microbiota in different treatment groups. PCoA = principal coordinate analysis, based on Bray-Curtis diversity metrics (PERMANOVA test). (c) Phylum-level composition of intestinal microbiota. (d) Genus-level composition of intestinal microbiota. (e) Linear discriminant analysis effect size analysis of intestinal microbiota. Linear discriminant analysis >2; P < 0.05. (f, g) Cluster heatmap and network analysis of the intestinal microbiota in mice (Spearman correlation). In the network, red lines represent positive correlations, blue represents negative correlation, and node diameters represent relative abundance.
Figure 3. Difference analyses of functional genes of intestinal microbiota and correlation between sn-2 palmitate dose and diversity, phylum- and genus-level composition, and functional genes of intestinal microbiota (scatter plots with fitted regression line and 95% CI bands). Control: blank control, water; Milk: solvent control, 0.05 g/mL nonfat milk powder solution; Low: 600 mg/kg BW of sn-2 palmitate solution; Medium: 1,800 mg/kg BW of sn-2 palmitate solution; High: 5,400 mg/kg BW of sn-2 palmitate solution. (a–c) Difference analysis of functional genes of intestinal microbiota predicated by PICRUSt2. Values were compared by t-test (false discovery rate <0.05) in STAMP software (version 2.1.3; Parks et al., 2014). (d, e) Gene copy numbers box plots of low abundance functional genes in b and c. (f, g) α-Diversity; (h) Phylum-level composition; (i–l) Genus-level composition; (m–o) Functional genes predicated by PICRUSt2 (https://github.com/picrust/picrust2). Statistical significance of correlation was determined using the Spearman method. Shaded error band around the regression line indicates 95% CI. The y-axes of f and g represent the diversity index; the y-axes of h–l represent the number of operational taxonomic units after homogenization; the y-axes of m–o represent the number of copies of the gene encoding the enzyme associated with the predicted function. The error bars represent SD. For box plots, upper and lower edges of boxes indicate the interquartile range, midlines indicate the median, lower and upper whiskers indicate the 95% CI, and dots indicate the outliers.
spiraceae, Mucispirillum, and Bacteroides. The relative abundance of Lachnospiraceae_NK4A136_group (phylum Firmicutes) decreased with increasing sn-2 palmitate dose (P = 0.0077, Figure 3i). The relative abundances of Bacteroides, uncultured_Lachnospiraceae, and uncultured_Muribaculaceae (phylum Bacteroidetes) increased with an increase in the sn-2 palmitate dose (Figure 3j–l).

In addition, linear discriminant analysis effect size was used to analyze the bacteria with differences between groups, namely biomarkers, as shown in Figure 2e. The number of bacterial communities with significant differences increased with increased sn-2 palmitate dose. The highest number of bacterial biomarkers in the sn-2 palmitate high-dose group mainly included Bacteroides, Prevotellaceae_UCG001, Weissella, Ruminococcaceae_NK4A214_group, Aerococcus, and Parabacteroides. Bifidobacterium and Dubosiella were significantly enriched in the sn-2 palmitate medium-dose group. Lachnospiraceae_NK4A136_group and Faecalibacterium were significantly enriched in the low-dose sn-2 palmitate group. These bacteria have been reported to produce butyrate (Louis and Flint, 2017). We also performed clustering and heatmap analysis and network graph analysis at the genus level. Figure 2f shows the clustering of the top 20 genera in terms of abundance in each group. In the sn-2 palmitate high-dose group, uncultured_Lachnospiraceae, Bacteroides, uncultured_Muribaculaceae, and Lachnoclostridium were clustered to a large extent. Alistipes, Ruminococcaceae_UCG014, and Lachnospiraceae_NK4A136_group clustered in the sn-2 palmitate medium-dose group. The sn-2 palmitate low-dose group was clustered into uncultured_Muribaculaceae, Lachnospiraceae_NK4A136_group, Coribacteriaceae_UCG002, unclassified_Ruminococcaceae, and Ruminococcaceae_UCG014. The results reflect not only the differences among groups but also the similarities among groups. A network diagram can be used to study the interaction relationships between each genus, with the red line representing a positive correlation, the blue representing a negative correlation, and node diameters representing relative abundance. As shown in Figure 2g, the central genera were Lachnospiraceae_NK4A136_group and unclassified_Ruminococcaceae, with many genera correlating with them, indicating their importance in the intestinal microbiota of mice.

The 16S rRNA gene sequencing data were predicted based on PICRUSt2 to analyze the composition of functional genes in the metabolic pathways of the intestinal microbiota of mice in different treatment groups. The differences in metabolic functional pathways at the third level of Kyoto Encyclopedia of Genes and Genomes between the milk powder control group and the sn-2 palmitate low-, medium-, and high-dose groups were analyzed using STAMP software (version 2.1.3; Parks et al., 2014). As shown in Figure 3a–c, the numbers of differentially predicted functional genes were positively correlated with the dose of sn-2 palmitate, respectively 6, 9, and 11 in the 3 dose groups (from low to high). Compared with the milk powder control group, the sn-2 palmitate groups were predicted to have a higher number of metabolic genes related to carotenoid biosynthesis, mineral absorption, isoflavonoid biosynthesis, biotin metabolism, glyoxylate and dicarboxylate metabolism, carbon fixation pathway, and phospholipase D signaling pathway. Furthermore, the numbers of genes encoding autophagy-yeast, phospholipase D signaling pathway, and pentose and glucuronate interconversion metabolic functions in the intestinal microbiota were predicted to increase with increased sn-2 palmitate dose (Figure 3m–o). The number of differential functional genes among 3 sn-2 palmitate experimental groups was highest between the high- and low-dose groups, second highest between the high- and medium-dose groups, and lowest between the medium- and low-dose groups, with 19, 13, and 4 of predicted metabolic pathways, respectively (Supplemental Figure S2; https://doi.org/10.6084/m9.figshare.23244389, Wang, 2023). The results imply that probably the intestinal microbiota genes function more similarly in the medium- and low-dose groups, and the high dose of sn-2 palmitate might regulate intestinal microbiota function. Compared with the medium- and low-dose groups, the high-dose group was predicted to have a greater number of metabolic genes related to lysosome, flavone and flavonol biosynthesis, α-linolenic acid metabolism, biosynthesis of siderophore group nonribosomal peptides, linoleic acid metabolism, and arachidonic acid metabolism. The potential effects of high-dose sn-2 palmitate on intestinal microbiota function may be induced by altering the relative abundance of specific taxa of the intestinal microorganisms.

**Effects of sn-2 Palmitate on Metabolites of Gut Microbiota**

We first focused on the effect of sn-2 palmitate on SCFA produced by intestinal microbiota. The results are shown in Figure 4a. The content of acetate was the highest in the intestinal tract of all mice, which is consistent with the previous report (Morrison and Preston, 2016). Among them, the acetate content in the sn-2 palmitate high-dose group (about 174.43 μmol/g) was significantly higher than that in the milk powder control group (about 97.05 μmol/g), indicating that sn-2 palmitate facilitates the production of acetate in the gut. Similar results were obtained for propionate,
among which the high-dose sn-2 palmitate group had the highest propionate content, 43.67 μmol/g. The butyrate content in the three sn-2 palmitate groups was not statistically different, but all were significantly higher than the milk powder control group. The isobutyrate in the high-dose group was the highest, at approximately 5.13 μmol/g, but it was not different from that in the milk powder control group. The feces of all mice were low in isovalerate and valerate and were not significantly different among the groups.

Furthermore, we analyzed metabolite species and relative levels in colon contents using nontargeted metabolomics to investigate the overall metabolic capacity of sn-2 palmitate on the intestinal microbiota. After merging the data in the positive and negative ion modes, the metabolites were analyzed by orthogonal projections to latent structures discriminant analysis using SIMCA software (version 14.0; http://www.umetrics.com/simca). As shown in Figure 4c, a clear separation existed between the groups, indicating that the metabolites of the intestinal microbiota in each group were significantly different. Annotated metabolites were clustered based on their structural similarities. The sn-2 palmitate low-dose and middle-dose groups were the closest in Figure 4c, indicating that the metabolites in these 2 groups were similar. Metabolite changes were more pronounced with increasing doses of sn-2 palmitate compared with the milk powder control group. The sn-2 palmitate intervention caused changes in the metabolites of the intestinal microbiota, of which the low dose had a significant effect.

In addition, we performed clustering and heatmap analysis of the metabolites of the mice gut microbiota. Figure 4b shows the clustering of the top 30 metabolites in terms of abundance and grouping. The sn-2 palmitate low-dose group clustered metabolites included 2-hydroxypropyl pentadecanoate, tiglic acid, lithocholic acid, and 3,3,5-trimethylcyclohexyl-1H-inden-1-yl. The sn-2 palmitate medium-dose group had the most clustered metabolites, including glycerophosphate-N-palmitoyl ethanolamine, 2-hydroxypropyl pentadecanoate, ethyl palmitylate, pentadecanoic acid, lithochoic acid, N-arachidonic glycerine, and 5-hydroxyindole-3-acetic acid. The sn-2 palmitate high-dose group showed increased levels of 2-hydroxypropyl pentadecane 2-hydroxypropyl pentadecanoate, glycerophosphate-N-palmitoyl ethanolamine, oleic acid alkyne, linoleic acid, and myristic acid. The abundance of 2-hydroxycinnamic acid and linoleic acid in the colon contents increased with an increase in the sn-2 palmitate dose (P = 0.044, Figure 4d; P = 0.0067, Figure 4e).

Metabolite changes may affect the physiological health of the host. The magnitude of this change can be measured by calculating the FC of metabolites, with an upregulated FC being positive and a downregulated FC being negative. As shown in Figure 4f–h, the yellow areas are metabolites that satisfy P < 0.5 and FC >2, indicating that these metabolites are significantly different among the groups. Compared with the milk powder control group, the sn-2 palmitate low-dose group significantly upregulated 9 metabolites and downregulated 8 metabolites (Figure 4f and Supplemental Table S1; https://doi.org/10.6084/m9.figshare.23244389, Wang, 2023); The medium-dose group significantly upregulated 16 metabolites and downregulated 12 metabolites (Figure 4g and Supplemental Table S2; https://doi.org/10.6084/m9.figshare.23244389, Wang, 2023). The high-dose group significantly upregulated 10 metabolites and downregulated 7 metabolites (Figure 4h and Supplemental Table S3; https://doi.org/10.6084/m9.figshare.23244389, Wang, 2023).

Pathway enrichment analysis was performed for different metabolites between the sn-2 palmitate and milk powder control groups. Figure 4i–k shows the metabolic pathways that were significantly affected (P < 0.05). Figure 4i shows that, at low dose, the significantly affected pathways were arginine and proline metabolism and aminoacyl-tRNA biosynthesis; Figure 4j shows that at medium dose, the significantly affected pathway was arginine and proline metabolism; Figure 4k shows that at high dose, the significantly affected pathway was purine metabolism.

**Spearman Rank Correlation Coefficient Analysis of Bacterial Genera and Metabolites**

To clarify the effects of sn-2 palmitate intervention on intestinal microbiota metabolism, we further analyzed the correlation between differential metabolites (variable importance in projection >1, FC >2, and P < 0.05) and high bacterial abundance (top 30 relative abundance). Notably, we found that alterations in the metabolic function of the intestinal microbiota might be induced by interactions among bacteria. As shown in Figure 5, the correlations between bacteria such as *uncultured_Muribaculaceae2*, *Lachnospiraceae A2*, *Alloprevotella*, *Lactobacillus*, *Ruminiclostridium*, and *uncultured_Bacteroidales*, and metabolites were consistent, which was opposite to *Lachnolactobacterium*, *Ruminococcaceae_UCG014*, *Butyrivimonas*, *uncultured_Ruminococcaceae*, *Mucispirillum*, *Lachnospiraceae_NK4A136_group*, *Roseburia*, *Coriobacteriaceae_UCG002*, *Prevotellaceae_UCG001*, *Bacteroides*, *Ruminococcus 1*, *Desulfovibrio*, and *Parabacteroides*. 2,3-Dihydroxypropyl 3,4,5-trihydroxybenzoate and hydroxycartecol were significantly positively (P < 0.05) correlated with *Lachnolactobacterium*, *Ruminococcaceae_UCG014*, *Butyrivimonas*, and *Lachnospirea*.
Figure 4. Effects of sn-2 palmitate on metabolites of intestinal microbiota. Control: blank control, water; Milk: solvent control, 0.05 g/mL nonfat milk powder solution; Low: 600 mg/kg BW of sn-2 palmitate solution; Medium: 1,800 mg/kg BW of sn-2 palmitate solution; High: 5,400 mg/kg BW of sn-2 palmitate solution. (a) Analysis of fecal short-chain fatty acids (SCFA) of mice in different treatment groups. One-way ANOVA followed by Tukey’s multiple comparisons test. The SCFA concentrations are expressed per gram of DM. Upper and lower dotted lines indicate the interquartile range; the dotted line in the center indicates the median. Significant differences ($P < 0.05$) among different treatments are indicated with different letters (a–c). (b) Cluster heatmap of metabolites of the intestinal microbiota in mice by k-means cluster analysis. The heatmap shows the clustering of the top 30 metabolites in terms of abundance. (c) Analysis of differential metabolites of the intestinal microbiota in mice. The significantly altered metabolites were detected with orthogonal projections to latent structures discriminant analysis (OPLS-DA) coefficient plots (variable importance in projection >1.0 and $P < 0.05$). (d, e) Scatter plots with fitted regression line and 95% CI bands, depicting a correlation between sn-2 palmitate dose and differential metabolites of intestinal microbiota. Statistical significance of correlation was determined using the Spearman method. Shaded error band around the regression line indicates the 95% CI. (f–h) Volcano map analyses of differential metabolites in the intestinal microbiota of mice ($t$-test adjusted by false discovery rate, FDR). FC = fold change. (i–k) Metabolic pathway analyses of differential metabolites in the intestinal microbiota of mice ($t$-test adjusted by FDR). (f and i) sn-2 palmitate low-dose group. (g and j) sn-2 palmitate medium-dose group. (g and k) sn-2 palmitate high-dose group.
Figure 5. Heatmap diagram of the Spearman rank correlation results between differential metabolites and high-abundance intestinal microbiota. *P < 0.05, **P < 0.01, ***P < 0.001. ESA = ethanesulfonic acid; MPBP = 4′-methyl-α-pyrrolidinobutiophenone; DOA = dioctyl adipate.
raceae_NK4A136_group but significantly negatively (P < 0.05) correlated with uncultured_Muribaculaceae2 and Lachnospiraceae_A2.

The high-dose of sn-2 palmitate group significantly upregulated fenpyroximate and downregulated 3β,16α-dihydroxy-5α-androstan-17-one of the colon contents of mice (Supplemental Table S3). Fenpyroximate was significantly positively (P < 0.05) correlated with Bacteroides, whereas 3β,16α-dihydroxy-5α-androstan-17-one was significantly negatively (P < 0.05) correlated with Bacteroides and uncultured_Lachnospiraceae (Figure 5). In addition, the relative abundance of Bacteroides and uncultured_Lachnospiraceae increased with an increase in the sn-2 palmitate dose (Figure 3j–k). Therefore, sn-2 palmitate might increase fenpyroximate level and decrease 3β,16α-dihydroxy-5α-androstan-17-one level in the mice intestine by stimulating the growth of Bacteroides and uncultured_Lachnospiraceae. These findings confirmed that sn-2 palmitate specifically regulated the intestinal microbiota and its metabolic capacity, affecting intestinal function.

**DISCUSSION**

This study investigated the effects of different doses of sn-2 palmitate on serum biochemical parameters, colonic pathology, diversity, composition, and metabolic function of the intestinal microbiota in mice. Approximately 70% of palmitic acid in HM fat is present at position sn-2, whereas unsaturated fatty acids are mainly connected at positions sn-1 and sn-3 (Kallio et al., 2017). The doses administered to mice were determined by conversion from infant intake. The results showed that doses in the experimental range did not cause abnormalities in colonic tissue pathology. No significant differences were observed in the serum biochemical indexes between the sn-2 palmitate experimental group and the solvent control group. These results are consistent with previous animal studies (Chen et al., 2022).

sn-2 Palmitate affected the diversity and composition of the intestinal microbiota. The results showed that high doses of sn-2 palmitate significantly modulated the α-diversity (evenness index and Shannon index) and β-diversity of the intestinal microbiota. Linear discriminant analysis effect size analysis revealed that different doses of sn-2 palmitate significantly regulated the beneficial bacterial genera that could increase SCFA, such as Bifidobacterium, Bacteroides, Weissella, Ruminococcus_NK4A214, Lachnospiraceae_NK4A136, and Faecalibaculum. Wang et al. (2020) also indicated that sn-2 palmitate could increase Bifidobacterium. However, they found that the group without sn-2 palmitate was enriched in Ruminococcaceae and Lachnospiraceae, which was different from our results. Chen et al. (2022) demonstrated that Faecalibaculum and Bifidobacterium were more abundant in the gut microbiota of the sn-2 palmitate group, which is consistent with our results. The differences among the studies might be due to differences in the experimental design. In general, this study showed that sn-2 palmitate could beneficially regulate the intestinal microbiota.

sn-2 Palmitate may have the potential to modulate the function of intestinal microbiota. According to the predicted gene function results, sn-2 palmitate groups were predicted to have a higher number of metabolic genes related to mineral absorption, carotenoid biosynthesis, isoflavonoid biosynthesis, biotin metabolism, glyoxylate and dicarboxylate metabolism, carbon fixation pathway, and phospholipase D signaling pathway, compared with the milk powder control group. However, these results need to be further validated. The up-regulation of mineral absorption function is consistent with the effects of sn-2 palmitate reported in previous studies (Yang et al., 2019; Wang et al., 2020; Zhang et al., 2022). The application of sn-2 palmitate in IF can effectively reduce the formation of calcium soaps of fatty acids and promote the absorption of calcium. A correlation between the relative abundance of SCFA-producing members of intestinal microbiome and the rate of calcium absorption has been observed in both in vivo and clinical studies, as well as with bone mineral density in animal models (Asemi and Esmaillzadeh, 2013; Weaver, 2015). Short-chain fatty acids actually lower the pH in the gastrointestinal tract, facilitating the solubility of calcium and consequently enhancing its transepithelial transport (Wawrzyniak and Suli-burska, 2021). Early studies indicated that some commensal bacteria such as Firmicutes to Bacteroidetes, Actinobacteria, Proteobacteria, and Verrucomicrobiota were able to synthesize carotenoid precursors (a form of 30 carbon [C30] carotenoids) in the lumen of the human gut, although the fate of these newly synthesized carotenoid precursors was unknown (Tian and Hua, 2010; Shindo and Misawa, 2014; Kanamoto et al., 2021; Misawa et al., 2022). From an evolutionary perspective, the expression of carotenoid biosynthesis may facilitate the survival and stress responses of the intestinal microbiota. Thus, we hypothesize that sn-2 palmitate may improve the function of the intestinal microbiota of infants. However, the functions of intestinal microbiota, such as mineral absorption and carotenoid biosynthesis, do not fully meet host needs. Future studies need to accurately distinguish gut microbe-produced micronutrients from dietary sources and verify whether gut microbiota-synthesized micronutrients are essential only for the function of local (or intestinal) tissues and how much these mechanisms contribute to host needs.
Short-chain fatty acids can be absorbed by the intestinal mucosa and act as signaling molecules between intestinal microbes and the host, as well as natural ligands for receptors in a range of cells and tissues (Koh et al., 2016; Morrison and Preston, 2016). Acetate, the most abundant SCFA in the gut, is produced from acetyl-CoA from glycolysis (den Besten et al., 2013), and the formation of butyrate and propionate occurs mainly through carbohydrate metabolism during glycolysis, but also through organic acids and amino acid metabolism proceeds (Louis and Flint, 2017). The results of our study show that sn-2 palmitate is beneficial for the production of SCFA in the gut early in life by increasing the relative abundance of SCFA-producing bacteria, consistent with previous studies (Hou et al., 2019). The SCFA play an important role in mucosal maintenance and integrity (Tang et al., 2015), affect a variety of tissues to improve glucose homeostasis in the gut, liver, and systemic circulation (Chambers et al., 2015), and promote the local and peripheral immune system of host metabolism through inflammatory pathways (Kimura et al., 2013). Acetate appears to stimulate leptin secretion in adipocytes, an important adipose-derived homeostatic signal that regulates energy balance and appetite. Propionate induces short-term appetite regulation through peptide tyrosine and glucagon-like peptide 1 (Chambers et al., 2015).

We analyzed the effects of sn-2 palmitate on intestinal microbiota metabolites using untargeted metabolomics. Orthogonal projections to latent structures discriminant analysis showed that sn-2 palmitate could regulate gut microbiota metabolites. Compared with the powder milk control group, 2-hydroxypropyl pentadecanoate, lithocholic acid, and glycerophosphate-N-palmitoyl ethanolamine were enriched in the sn-2 palmitate groups. Lithocholic acid is a representative secondary bile acid that plays a conductive role in maintaining homeostasis of the bile secretion pathway (Chen et al., 2020a). In addition, the metabolic pathways that sn-2 palmitate significantly affected were arginine and proline metabolism, aminoacyl-tRNA biosynthesis, and purine metabolism. Dysregulation of arginine metabolism and depletion of key arginine metabolites may be associated with many diseases, including obesity, metabolic syndrome, and asthma (Liao et al., 2020). Proline metabolism is key to energy production, redox homeostasis, signaling, and protein synthesis, especially the metabolism of collagen; nearly 25% of amino acids are proline (Guo et al., 2020). Aminoacyl-tRNA biosynthesis links tRNAs to their cognate amino acids for protein biosynthesis (Ji et al., 2017).

Previous studies have shown that sn-2 palmitate modulates the intestinal microbiota composition (Wan et al., 2017; Wang et al., 2020). Our findings suggest that dietary sn-2 palmitate mediates induces in intestinal microbiota diversity, structure, and metabolic function. In addition, we showed that different doses of sn-2 palmitate have different effects. The relative abundance of the phylum Bacteroidetes, Bacteroides, uncultured_Lachnospiraceae, and uncultured_Muribaculaceae and the number of genes of the intestinal microbiota predicted to have metabolic functions related to autophagy-yeast, phospholipase D signaling pathway, and pentose and glucuronate interconversions increased with increasing doses of sn-2 palmitate. In contrast, the relative abundance of Lachnospiraceae_NK4A136_group decreased with increasing doses of sn-2 palmitate. In addition, the abundance of 2-hydroxyxynamic acid and linoleic acid in the colon contents increased with an increase in sn-2 palmitate. The intervention with sn-2 palmitate shifted the overall metabolic profiles of the intestinal microbiota in mice and affected many microbiota-related metabolic pathways that are closely related to host physiology. The changes in intestinal microbiota that occurred in this study may be due to the supplementation of sn-2 palmitate altering the intake of fatty acids or the bioavailability of fatty acids. It may also be the result of a combination of multiple interactions following sn-2 palmitate ingestion, such as altered intestinal microenvironment, altered host immune levels, microbial cross-feeding, or competition in the gut. Our work contributes toward understanding the physiological significance of saturated fatty acids bound to the sn-2 position in HM and provides a reference value for bridging the gap between IF and HM.

CONCLUSIONS

This study showed that sn-2 palmitate affected intestinal microbiota and metabolic function. The α-diversity of the intestinal microbiota was greater in the high-dose sn-2 palmitate group than in the medium- and low-dose groups. The relative abundance of Bacteroidetes was positively correlated with sn-2 palmitate dose. At the bacterial genus level, the relative abundance of Lachnospiraceae_NK4A136_group was negatively correlated with dose, whereas the abundances of Bacteroides, uncultured_Lachnospiraceae, and uncultured_Muribaculaceae were positively correlated with sn-2 palmitate dose. sn-2 Palmitate significantly regulated SCFA produced by beneficial bacterial genera, increasing fecal levels of acetate, propionate, and butyrate. The numbers of genes potentially encoding phospholipase D signaling pathway, and pentose and glucuronate interconversion metabolic functions of intestinal microbiota increased with increasing doses of sn-2 palmitate. These findings suggest that sn-2 palmitate plays an important role in the intestinal microbiota and metabolism early in life.
Moreover, these results further emphasize the importance of fat structures in HM and IF.

ACKNOWLEDGMENTS

This work was supported by the National Key Research and Development Project (No. 2022YFD2100703; Beijing, China); the National Natural Science Foundation of China (No. 32021005 and 32122067; Beijing, China); the Fundamental Research Funds for the Central Universities JUSRP22013 (Jiangsu, China); and the “Bai-Qian-Wan Engineering and Technology Master Project” (Grant No. 2020ZX07B01, funded by the Government of Heilongjiang Province of the People’s Republic of China). Data and supplemental materials are available on Figshare (https://doi.org/10.6084/m9.figshare.23244389). The authors have not stated any conflicts of interest.

REFERENCES


ORCIDS

S. Wang https://orcid.org/0000-0003-1539-6072
W. Chen https://orcid.org/0000-0003-3438-4710
Q. Zhai https://orcid.org/0000-0002-0695-2675