



Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: <https://www.sciopen.com/journal/2097-0765>

Synergistic Enhancement of Vitamin D3 and Vitamin K1 Bioavailability via Medium- and Long-Chain Triglycerides: Micelle-Mediated Absorption, Gut Microbiota and Bone Density

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ABSTRACT: Vitamin D (VitD) and vitamin K (VitK), essential fat-soluble micronutrients regulating bone homeostasis and gut microbiota, exhibit high global deficiency rates. Infant formulas demonstrate low bioavailability of VitD3 and VitK1. This study investigated how medium- and long-chain triacylglycerols (MLCTs, MLCT1 vs MLCT2) with distinct MLCT concentrations (25% vs 40%) affect VitD3/VitK1 bioavailability using integrated *in vitro-in vivo* models. *In vitro* digestion revealed that 40% MLCT2 significantly enhanced free fatty acid release (52.81%, $P < 0.05$) and bioaccessibility (VitD3: 54.37%; VitK1: 67.14%, $P < 0.05$) compared to MLCT1. Pharmacokinetic analysis demonstrated that 40% MLCT2 achieved superior bioavailability (AUC: VitD3 3880.26 $\mu\text{g/L}\times\text{h}$; VitK1 710.12 $\mu\text{g/L}\times\text{h}$) and metabolic efficacy (serum 25OHVitD3: $(34.72 \pm 10.85) \text{ ng/mL}$; VitK1: $(27.28 \pm 2.5) \text{ ng/mL}$) compared to 25% MLCT2. Long-term animal studies showed 40% MLCT2 administration significantly improved bone mineral density ($(0.10 \pm 0.02) \text{ g/cm}^3$, $P < 0.001$) and enriched *Bacteroidetes* and *Eubacterium_coprostanoligenes_group* - taxa linked to vitamin metabolism. These findings establish MLCTs as advanced lipid carriers to combat VitD/VitK deficiencies, offering transformative strategies for nutrient delivery systems with enhanced bioavailability.

Keywords: Medium- and long-chain triglycerides, Fat-soluble vitamins bioavailability, Gut microbiota, Bone mineral density

1. Introduction

Human milk is widely recognized as the optimal infant food, yet it falls short of providing adequate vitamin D (VitD) and vitamin K (VitK). Infant formula serves as a crucial substitute when breastfeeding is unfeasible due to insufficient milk supply or social constraints. VitD, a fat-soluble vitamin integral to calcium-phosphorus homeostasis and skeletal health, has its nutritional status best gauged by serum 25 - hydroxyvitamin D3 (25OHVD3)¹. VitD deficiency manifests as inadequate bone mass and rickets in children, while in adults, it presents as reduced bone mineral density (BMD) and increased fracture susceptibility²⁻⁴.

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Received 17 June 2025
Received in revised form 9 October 2025
Accepted 20 January 2026

BMD can reflect the actual development of bones, and its content is related to the body's bone strength, so it is often used to assess the health of the human body⁵. Given the rapid skeletal growth in infancy, VitD deficiency is prevalent among growing infants⁶. Despite breastfeeding mothers' intake of prenatal vitamins and VitD-rich foods, infants remain at risk⁷. Alarming studies indicate that in our country, 72.8% of newborns suffer from moderate to severe VitD deficiency⁸, underscoring the urgent need for effective VitD supplementation strategies in infant nutrition.

Vitamin K, including naturally occurring fat-soluble - K1 and K2, and synthetic water-soluble K3 and K4, has vital functions^{9, 10}. Adults can synthesize VitK in the gut to meet physiological needs, but newborns cannot⁹. They're relatively VitK-deficient at birth, and their sterile gut initially can't generate enough VitK via microbiota. Exclusively breastfed infants often lack adequate VitK. Nearly 100% of newborns experience a physiological VitK concentration drop in plasma within 24 hours of birth. Unchecked, this decline heightens the risk of severe bleeding, including intracranial hemorrhage¹¹.

Existing approaches to improve Fat-soluble vitamins bioavailability primarily focus on three technical domains¹²: (1) solubility enhancement through physicochemical modulation, (2) controlled-release nanocarriers utilizing protein-polysaccharide matrices, and (3) mucosal-penetrating systems such as self-emulsifying formulations. While these methods demonstrate enhanced *in vitro* bioaccessibility, they fundamentally fail to address the biological barriers governing intestinal vitamin transport, particularly the molecular-level absorption limitations involving nuclear receptor interactions and transporter-mediated uptake mechanisms. The intestinal absorption of fat-soluble vitamins involves multiple critical steps, commencing with the release of vitamins from the food matrix and its subsequent incorporation into mixed micelles composed of phospholipids, cholesterol, bile salts, and lipid digestion products. Following gastrointestinal processing, these mixed micelles are recognized and transported across the intestinal epithelium through specialized cellular uptake mechanisms¹³. As crucial fat-soluble nutrient carriers, lipids are vital for VitD and VitK digestion and absorption across all stages¹⁴. MLCT defined by Tinglan¹⁵ as triglycerides with both medium-chain fatty acids (MCFA) and long-chain fatty acids (LCFA) on glycerol skeletons, are categorized into the medium-medium-long chain (MML) and medium-long-long chain (MLL) types based on the medium-chain fatty acid count. MLCT combines the advantages of LCT and MCT: it can provide energy quickly and provide long-chain essential fatty acids, and simultaneously release and transport MCFA and LCFA, avoiding the large production of MCFA, causing excessive ketone poisoning, and overcoming the defects of LCT in metabolic and immune function¹⁶. Wang et al.¹⁶ demonstrated long-chain triglycerides (LCT) are better than medium-chain triglycerides (MCT) for encapsulating and delivering fat-soluble vitamins. Guo Yiwen's¹⁴ study highlighted that STG-type MLCT outperforms MCT/LCT-type MLCT in delivering fat-soluble nutrients. Although MLCT's role in VitD3 digestion and absorption has been studied, little is known about how varying MLCT contents influence VitD3 and VitK1 digestion and absorption *in vivo* and *in vitro*, or its link to gut flora changes.

MLCT, naturally occurring in dietary fats such as breast milk (20 – 40% of total lipids¹⁵, exhibits distinct compositional profiles compared to infant formula. Current formulations also face limitations in VitD3 and VitK1 bioavailability. To bridge this gap, we propose a dual-focused strategy: (1) synthesizing breast milk-mimetic MLCTs through enzymatic esterification, and (2) systematically evaluating dose-dependent MLCT effects on VitD3 and VitK1 bioavailability *in vitro* (infant *in vitro* digestion simulation) and *in vivo* (SD rats model), with parallel analysis of gut microbiota modulation via 16S rRNA sequencing. This work advances the rational design of infant nutrition lipids to potentiate micronutrient absorption and lays the foundation for the subsequent development of similar products.

2. Materials and methods

2.1 Chemicals and reagents

The VitD3/VitK1 and LCT were obtained from Yili Co., Ltd (Inner Mongolia, China).

Pepsin from porcine pancreas (P110928) was purchased from Aladdin (Shanghai, China) and was of porcine origin. Lipase A “Amano” from *Aspergillus niger* was obtained from Amano-enzyme (Aichi, Japan). Pancreatin from porcine pancreas (P7545 8XUSP) was purchased from Sigma-Aldrich (St Louis, MO). Pig bile salt was purchased from Solarbio (Beijing, China). All other chemicals used were of analytical grade and were obtained from Sigma-Aldrich.

2.2 Preparation and purification of the different MLCT structures

MLCT was synthesized via transesterification according to the optimized method previously established by Zhao Manli et al. in our research group¹⁷. HORO was mixed with MCT and/or coconut oil at a molar ratio of 1:1. Subsequently, 10 wt% (based on total substrate weight) of the immobilized lipase Lipozyme RM IM was added, and the reaction mixture was incubated at 60°C for 3 hours with shaking (200 rpm), yielding MLCT1 and MLCT2.

The crude product was purified using molecular distillation, following the methodology detailed by Du Yingxue et al. in our previous work¹⁸.

2.3 Composition analysis of MLCT

2.3.1. Triglyceride composition analysis

MLCT was accurately weighed, dissolved in acetonitrile-isopropanol (1:9) to a concentration of 3 mg/mL, filtered through a 0.22 μ m filter membrane, and detected by UPLC-Q-TOF. Details of the chromatography and mass spectrometry conditions used in the analysis are described in previously published method^{19, 20}.

2.3.2. Total fatty acid analysis

Fatty acids were determined by acid methyl esterification method²¹, taking 2mg rude lipid, then adding 2mL 14% boron trifluoride-methanol solution and 1mL toluene, filling with nitrogen, sealing, heating 60min in a water bath at 90 °C, cooling to room temperature, then adding n-hexane 1mL distilled water of 3mL, mixing, centrifuging 10min at 4000r, taking the supernatant, drying with nitrogen, adding 1mL n-hexane re-dissolution, filter membrane, injection into the sample bottle, GC machine analysis.

2.4. Preparation of emulsions for simulating *in vitro* digestion

The two MLCTs are compounded with LCT according to the composition of TAG in breast milk, so that the proportion of MCLT is 25% and 40%, respectively. Then, under the condition of avoiding light and 50–55°C, the stirring speed is 100 r/min, stir for 30 min, mixing evenly, cooling to 37°C, adding VitD3 and VitK1, continuing to stir 20 min to mix well, and then four VitD3 and VitK1 oil compositions are obtained, namely, 25% MLCT1: 25% MLCT1+VitD3+VitK1; 40% MLCT1: 40% MLCT1+VitD3+VitK1; 25% MLCT2: 25% MLCT2+VitD3+VitK1; 40% MLCT2: 40% MLCT2+VitD3+VitK1.

At the same time, take 1% of the emulsifier (whey protein/casein, 7/3) and stir in a water bath at 37 °C for 30 minutes. Add 3wt% of the above-mentioned VitD3 and VitK1 MLCT composition (the final VitD3 and VitK1 account for 1% of the oil) and continue to stir and mix well, then use a high-speed dispersing homogenizer (FSH-2A, Jiangsu Jinyi Instrument Technology Co., Ltd.) with a shear rate of 10,000 rpm, shear for 5 minutes, and repeat 3 times. Finally, a stable oil-in-water emulsion was obtained to prepare for subsequent *in vitro* digestion.

2.5. *In vitro* digestion assay

2.5.1. Preparation of digestion solution

In order to simulate the digestive system of infants, the preparation of simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) refers to Ménard et al.²² method. SGF included: 94 mM sodium chloride and 13 mM potassium chloride, SIF included: 164 mM sodium chloride, 10 mM potassium chloride, 85 mM sodium bicarbonate, and 119 mM calcium chloride.

2.5.2. Simulated gastrointestinal digestion

The *in vitro* digestion model in this study was established according to Ménard et al.²², with minor modifications. The emulsion was mixed with SGF at the ratio of 63:37 (v/v), and the system pH was then adjusted to 5.3 using HCl. Porcine pepsin and lipase from *Aspergillus niger* were added into the final digestion mixture to achieve final concentrations of 268 U/mL and 19 U/mL, respectively. The mixture was incubated in a shaking water bath (50 rpm, 37 °C) for 60 min, and samples were collected at 0 (G0), 30 (G30), and 60 (G60) minutes for subsequent analysis.

At the intestinal digestion stage, SIF was added according to the volume ratio of emulsion to gastrointestinal fluid at 39:61, to replicate the physiological environment of the infant small intestine. The pH was adjusted to 6.6 using 0.5 M HCl. Pancreatin and bile salts were then added to the final digestion mixture at concentrations of 300 U/mL and 3.1 mM. The mixture was incubated in a shaking water bath (50 rpm, 37 °C), and the pH was maintained at a constant value of 6.6 using 0.1 M NaOH. Samples were collected at 30 (I30), 60 (I60), and 120 (I120) minutes during this phase, and the inhibitor was added immediately to stop the reaction²³.

2.5.3. Particle size

The particle size of the emulsion was measured using Malvern Zetasizer Nano ZS90. Before the measurements, the samples were diluted 25 times with PBS (for stomach, pH = 5.3; for intestinal, pH = 6.5,). The particle size test was performed according to the refractive index of oil of 1.333, and the average value was taken after three repetitions.

2.5.4. Zeta-potential

The zeta potential of the emulsion was measured using Malvern Zetasizer Nano ZS90, the average value was taken after three repetitions.

2.5.5. Microstructure

The structure and morphology (Christelle et al., 2011; Ling et al., 2023) were served by a laser confocal microscope^{24, 25}. The lipids were stained with Nile red fluorescent dye. About 10 μ L Nile red solution (excitation wavelength = 488nm) was added to the 0.1mL sample to avoid light staining above 30min and observed on the machine.

2.5.6. Determination of free fatty acids

During simulated digestion *in vitro*, NaOH consumption in the gastrointestinal system was recorded by titration, and the amount of fatty acid released was calculated by formula²³:

$$\text{FFA Release rate} = \frac{C_0 \times V_t \times 0.001 \times M}{3m} \times 100\%$$

In the formula: C_0 is the concentration of NaOH (mol/L); V_t is the total volume of NaOH solution consumed (mL); m is the quality of lipids in the emulsion(g); M is the average molar mass of lipids (g/mol).

2.5.7. Bioaccessibility

Extraction concerning the patented method²⁶, Collect the digestion products at different stages of the small intestine and centrifuge them at 4°C, 12000 r/min for 45min; after centrifugation, divided into three phases, the top is the oil layer, the middle is the micelle phase, and the bottom is the dense insoluble matter. Aspirate the micelle phase and the digestion products of the small intestine and add a mixed solution of n-hexane-anhydrous ethanol (1:1) in a ratio of 1:2, vortex for 2min, centrifuge at 4000r/min for 10min, transfer the supernatant phase, and blow dry with N₂. Add 0.6 mL chloroform-methanol (2:1) to dissolve again and filter the membrane. LC-MS/MS detection. Calculate according to the following formula:

$$\text{Bioaccessibility (\%)} = \frac{C_{\text{Micelles}}}{C_{\text{Total}}} \times 100 \quad (4.3)$$

Note: C_{micelle} refers to the concentration of vitamins in the middle layer obtained after centrifugation after small intestinal digestion, and C_{total} refers to the concentration of vitamins extracted from the small intestinal digestion products.

2.5.8. Extraction and detection of digestible lipids

Extraction of lipids from digestion products by chloroform-methanol method²⁶, the composition of lipids before and after digestion was detected by LC-MS/MS. 5 mL of digestion solution and 10 mL of methanol solution were taken, vortexed and extracted for 2-3 minutes, and then 5 mL of ultrapure water and 5 mL of

chloroform were added, and vortexed and extracted again for 1 minute. After centrifugation at 4200 rpm/min for 10 minutes, the lower chloroform layer was taken and dried with nitrogen for analysis.

Detection method: The target compound was separated by ultra-high performance liquid chromatography (UHPLC) on a Hypersil GOLD VANQUISH (2.1 μm , 100 mm, 1.9 μm) liquid chromatographic column. The mobile phase A was acetonitrile-aqueous solution (6:4,v/v), and the mobile phase B was acetonitrile-isopropanol solution (1:9,v/v), containing 10 mM ammonium formate, respectively. Elution gradient: 0-2 min, mobile phase B is 30%, 2-25 min, mobile phase B increases from 30% to 100%, 25-35 min, mobile phase B decreases from 100% to 30%. The flow rate of the mobile phase is 0.3 mL/min, the column temperature is 45 °C, and the injection volume is 2 μL .

Mass spectrometry analysis was performed on a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA), and each sample was detected in positive and negative ion modes using an electrospray ionization (ESI) source. The conditions for the positive ion mode were as follows: heater temperature, 300°C; sheath gas flow rate, 45 arb; auxiliary gas flow rate, 15 arb; scan gas flow rate, 1 arb; spray voltage, 3.0 kV; capillary temperature, 350°C; S-Lens RF level, 50%; scan range, 200-1800 m/z. The conditions for the negative ion mode were the same as those for the positive ion mode, except that the spray voltage was 2.5 kV; the S-Lens RF level, 60% was different. Masslynx4.1 software was used for mass spectrometry data acquisition, and Xcalibur software and a lipid secondary database were used for lipid identification.

2.5.9. Detection of VitD3 and VitK1 in digested samples

The liquid phase conditions were slightly modified according to the national standard method^{27, 28}: C18 column (2.1 mm $\times$ 100 mm $\times$ 1.8 μm ,), column temperature: 40 °C. Mobile phase A, 0.05% formic acid solution, mobile phase B, 0.05% formic acid-methanol solution. The elution gradient was set as follows: 0-1 min, 80% B; 2-5.8 min, 80-94% B; 6-11 min, 100% B; and 12-14 min, 80% B. Flow rate: 0.3 mL/min, injection volume: 2 μL .

Mass spectrometry conditions: ionization mode: ESI+, sheath gas temperature: 375 °C, sheath gas flow rate: 12 L/min, nozzle voltage: 500 V, atomizer pressure, 172 kPa, capillary voltage: 4500 V, drying gas temperature: 325 °C, dry gas flow rate: 10 L/min, relative molecular weight scanning range (m/z) 50-500.

2.6 Animal experiment

2.6.1. Designs of Animal Experiments and Diets.

48 male newly weaned SPF SD rats (3 weeks old), weighing 50-70g, were purchased from Weitong Lihua Animal Co., Ltd., with experimental animal production license SCXK (Zhejiang) 2019-0001, and were kept in the Animal Experiment Center of Nanchang University. The breeding environment was SPF grade, the ambient temperature was (24 $\pm$ 2°C), the humidity was 50-60%, and the light was 12h/12h day and night cycle. The use of experimental animals complies with the "Basic Management Regulations for Animal Experiments of Nanchang University" and has been approved by the "Nanchang University Experimental Animal Ethics Committee" (approval number: NCULAE-20231226001).

In the pharmacokinetic experiment, the rats were randomly divided into two groups according to their body weight, half male and half female: 25% MLCT2 group and 40% MLCT2 group, with 6 rats in each group. The feed was the AIN93-G standard formula feed. After one week of adaptive feeding, the above nutrient oil was used for gavage. The doses of VitD3 and VitK1 were both 100 µg/kg body weight, after gavage, blood was collected through the eye socket with a 1.5mL EP tube at different times. About 300µL of blood samples were collected^{29, 30}.

In the long-term experiment, the animals were randomly divided into three groups according to their body weight: soybean oil control group, 25% MLCT2 groups (25% MLCT2 + 75% LCT), and 40% MLCT2 groups (40% MLCT2 + 60% LCT), with 12 animals in each group. The feed was prepared based on the AIN93-G standard formula, with modified oil composition and VitD3 content. The specific formulas of each group are shown in Table S4. The animals were allowed to take feed and water freely, and the food intake was measured. The animals were fed for 4 weeks. The animal grouping and flow chart are shown in Fig. 4A. After the experiment, the rats were euthanized using carbon dioxide. The protocol has been approved by the Institutional Animal Ethics Committee. The eyeballs were removed to collect blood, bones, intestinal tissues, cecal feces, and liver, kidney, and other samples were frozen in an ultra-low temperature refrigerator at -80°C.

2.6.2. Pharmacokinetics Study of VitD and VitK

Pharmacokinetic parameters were determined using Drug and Phoenix 8.1 software to calculate the following: total area under the curve (AUC), mean residence time (MRT), time to peak concentration (Tmax), and peak concentration (Cmax).

2.6.3. Serum 25OHVD and VitK concentration

The concentrations of serum 25OHVD and VitK1 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Xinle Biotechnology Co., Ltd., China).

2.6.4. Serum biochemical index

TG, TC, LDL-C, and HDL-C were measured using an automatic biochemical analyzer.

2.6.5. Detection of bone and femoral tissue parameters

The femurs of SD rats were collected and fixed with 4% para-formaldehyde. The femoral bone tissue of the rats was detected using a Micro-CT scanner. The resolution was set to 10 µm. After setting the other corresponding parameters, a scan was performed and the distal femoral trabecular area of the rats was selected.

2.6.6. Intestinal flora sequencing

Collect cecal feces for intestinal flora analysis. 16S rDNA amplicon sequencing usually selects a specific region, uses primers for PCR amplification, and then performs sequencing analysis and bacterial species identification. A small fragment library is constructed according to the characteristics of the amplified region, and the library is sequenced at both ends based on the Illumina NovaSeq sequencing platform. After Reads splicing and filtering, OTUs (Operational Taxonomic Units) clustering or ASVs (AmpliCon Sequence Variants) denoising, the valid data is then annotated with species and analyzed for abundance, thereby revealing the

species composition of the sample, and further α -diversity and β -diversity analysis can not only explore the differences in community structure between samples but also perform personalized analysis and in-depth data mining according to project requirements.

The cecal content samples of rats in the control group, 25% MLCT2 group, and 40% MLCT2 group were collected for 16SrRNA gene sequencing, which was commissioned to Beijing Novogene Technology Co., Ltd. According to the manufacturer's protocol, total genomic DNA was first extracted. PCR amplified the V3-V4 hypervariable region of the bacterial 16SrRNA gene.

2.7. Statistical analysis

All experiments were repeated three times. One-way ANOVA followed by Tukey's test (SPSS20.0 software) was used to analyze significant difference, and Origin and Graphprism drawing software were used for drawing.

3. Results

3.1. Compositional Characteristics of MLCT Lipid

Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) revealed distinct triglyceride profiles in MLCT1 and MLCT2. Based on positional distribution patterns, MLCTs were classified into MML and MLL subclasses.

Comparative analysis demonstrated significant compositional differences between MLCT formulations (Fig. 1A). MLCT1 contained 5.56% MCT, 64.08% MLCT, and 30.35% LCT, with MML and MLL subclasses constituting 27.51% and 36.56% of total lipids, respectively (Fig. 1B). The predominant MLCT species in MLCT1 were identified as Ca/O/L (6.69% of MLCT, 4.29% total lipids), Cy/O/O (6.57% of MLCT, 4.21% total lipids), and Ca/S/L (6.34% of MLCT, 4.06% total lipids) (Table S1).

In contrast, MLCT2 exhibited reduced MCT content (3.16%) with 61.74% MLCT and 35.10% LCT. The MML and MLL distribution shifted to 21.25% and 40.48%, respectively (Fig. 1B). Notably, MLCT2 showed enhanced proportions of Ca/L/L (8.30% of MLCT, 5.12% total lipids), Ca/O/L (7.30% of MLCT, 4.51% total lipids), and Cy/L/Ln (6.43% of MLCT, 3.97% total lipids) (Table S1).

Gas chromatography (GC) analysis revealed significant fatty acid profile variations (Table S2). MLCT1 contained predominant LCFAs: C18:1 ($30.9 \pm 1.35\%$), C18:2+C18:3 ($39.12 \pm 1.86\%$), and C16:0 ($14.34 \pm 0.16\%$), with MCFAs primarily comprising C8:0 ($2.01 \pm 1.07\%$) and C10:0 ($5.78 \pm 2.46\%$). Conversely, MLCT2 displayed elevated polyunsaturated fatty acids (C18:2+C18:3: $49.06 \pm 1.42\%$) with reduced C16:0 ($9.45 \pm 0.51\%$) and trace C8:0 ($0.04 \pm 0.01\%$).

Critical compositional divergences emerged through comparative analysis: MLCT2 demonstrated 9.9% higher C18:2+C18:3 content ($P < 0.05$) and 4.9% lower C16:0 levels compared to MLCT1, alongside a 2.0% reduction in C18:1. These findings highlight formulation-dependent variations in fatty acid distribution and triglyceride architecture.

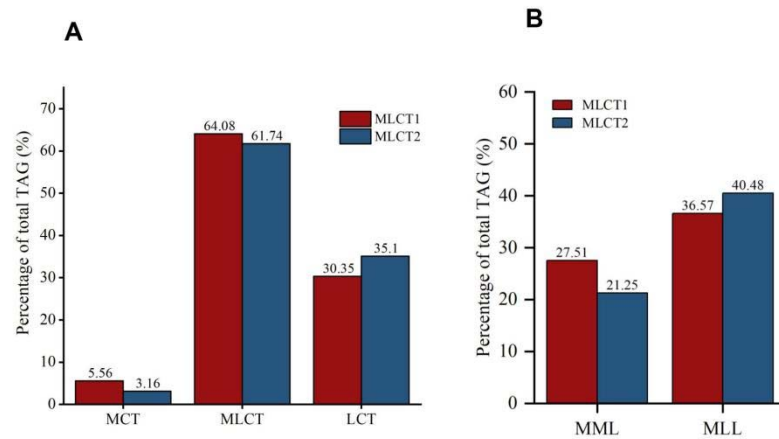


Figure 1. Analysis of triglyceride composition of two MLCT

3.2. Physical characteristics of digestion products of MLCT

In the initial phase (G0), homogeneous emulsion distribution with uniform lipid droplet sizing (25% MLCT: D4,3 were 650-660 nm, 40% MLCT: D4,3 were 470-540 nm). In the gastric phase (G60), protein-coated lipid droplets aggregated significantly ($D_{4,3} > 1100$ nm), attributable to gastric acid-induced isoelectric precipitation and pepsin-mediated interfacial protein hydrolysis (Ling et al., 2023). In the intestinal phase (I120), pronounced droplet fragmentation ($D_{4,3} < 300$ nm) confirmed structural lipid hydrolysis, consistent with confocal microscopy observations (Figure 2A).

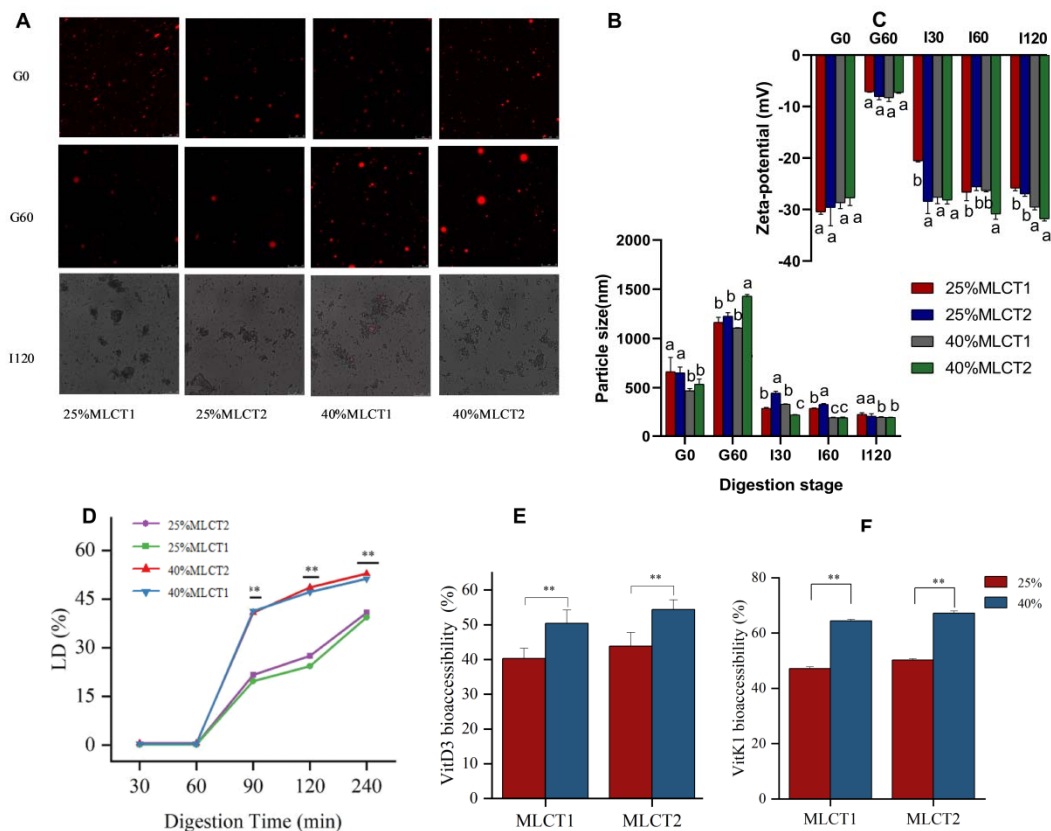


Figure 2. Micro-structure during digestion (A); particle size (B); Zeta potential (C); degree of lipolysis (D); VitD3 and VitK1 bioaccessibility (E-F).

Particle size dynamics (Fig. 2B) revealed gastric-stage instability across formulations, with 40% MLCT2 exhibiting maximum flocculation ($D_{4,3} = 1.43 \pm 160$ nm). Post-intestinal processing showed size hierarchy: 40% MLCT2 (200 nm) < 40% MLCT1 (200 nm) < 25% MLCT2 (210 nm) < 25% MLCT1 (230 nm), though without statistical significance.

Zeta potential monitoring (Fig. 2C) demonstrated that high negative charge (-30.5 ± 0.42 mV) from interfacial at initial stage, charge reduction to -7.12 ± 0.01 mV at G60 due to proton screening effects at gastric stage, charge recovery followed the sequence 40% MLCT2 (-31.8 mV) > 40% MLCT1 (-29.55 mV) > 25% MLCT2 (-27.00 mV) > 25% MLCT1 (-25.85 mV) ($P < 0.05$), correlating with lipase-mediated interface restructuring in intestinal stage.

3.3. The hydrolysis kinetics of two MLCT formulations

The hydrolysis kinetics of two MLCT formulations were quantified through free fatty acid (FFA) release monitoring, as shown in Fig. 2D. During gastric digestion, minimal FFA liberation (<1%) was observed. Intestinal digestion revealed significantly enhanced hydrolysis kinetics, with distinct formulation-dependent patterns. At 30-minute incubation, bile salt-mediated emulsification facilitated lipase-substrate interaction, resulting in rapid FFA release. The 40% MLCT formulations demonstrated superior hydrolysis (40.80%-41.32%) compared to 25% MLCT counterparts (19.66%-21.65%). This differential persisted through 120-minute digestion, showing the following hierarchy: 40% MLCT2 > 40% MLCT1 > 25% MLCT2 > 25% MLCT1. Final FFA liberation rates reached 51.28%-52.81% for 40% MLCT versus 39.33%-40.81% for 25% MLCT formulations ($P < 0.05$).

3.4. Effects of MLCT on the bioaccessibility of VitD3 and VitK1

As shown in Fig. 2E, the bioaccessibility of VitD3 in 40% MLCT2 (54.37%) was the highest, followed by 40% MLCT1 (50.43%), 25% MLCT2 (43.82%) and 25% MLCT1 (40.26%). The bioaccessibility of VitK1 was ranked: 40% MLCT2 (67.14%) > 40% MLCT1 (64.41%) > 25% MLCT2 (50.24%) > 25% MLCT1 (47.14%) (Fig. 2F). The bioaccessibility of 40% MLCT VitD3 and VitK1 was significantly higher than that of 25% MLCT. It can be concluded that the more the content of MLCT in oil composition, the higher the bioaccessibility of VitD3 and VitK1.

3.5. Changes of lipids during in vitro digestion of MLCT

Comparative analysis revealed that while MLCT1 and MLCT2 exhibited comparable in vitro digestibility ($p > 0.05$), MLCT2 demonstrated significantly enhanced bioaccessibility of VitD and VitK. This observation prompted a focused investigation of MLCT2's digestion products to elucidate structural determinants influencing vitamin solubilization.

During initial and gastric phases, triacylglycerols (TG) and diacylglycerols (DG) predominated, with minimal monoacylglycerol (MG) generation (<1%) (Fig. 3A, B; Table S3). During the intestinal digestion stage, TG and DG are further digested by pancreatic lipase (sn-1,3-specific), yielding FFA, MG18:1, and MG18:2 as terminal products. There were significant formulation-dependent differences emerged in free fatty

acid composition: 40% MLCT2 produced $56.84 \pm 1.18\%$ C18:2 (vs $47.62 \pm 1.14\%$ in 25% MLCT2) with a concomitant reduction in C18:1 ($31.90 \pm 0.20\%$ vs $38.29 \pm 1.24\%$, $P < 0.05$).

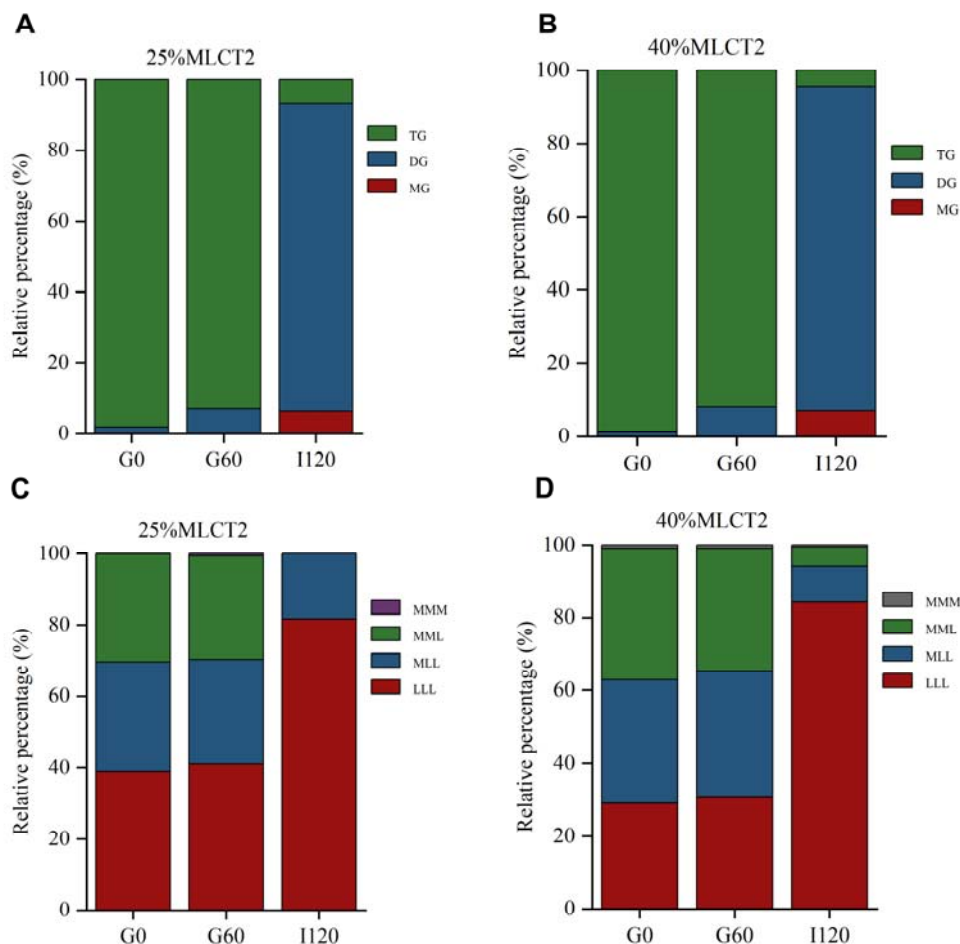


Figure 3. The percentage of lipids (A,B), triglyceride length (C,D) were distributed during in vitro digestion

Pre-digestion characterization showed 40% MLCT2 contained more MLL/MML-type TG (33.77%/36.21%) compared to 25% MLCT2 (30.45%/30.24%) (Fig. 3C, D), post-digestion analysis revealed concentration-dependent hydrolysis efficiency, with 40% MLCT2 exhibiting lower residual TG (4.52% vs 6.63%) and reduced MLL retention (9.78% vs 18.29%), which revealed that 25% MLCT contained more unhydrolyzed MLL type TAG, this was also consistent with the fact that 25% MLCT contains less C18:2. It is worth noting that, the remaining undigested triglycerides were mainly LLL (TG 16:0/18:1/18:1, TG 18:1/18:2/18:2, TG 18:2/18:2/18:2).

3.6. The fatty acid composition of feed

GC characterization revealed distinct fatty acid profiles across experimental diets (Tables S4-S5). The soybean oil-based control diet predominantly contained unsaturated fatty acids, with C18:1 ($33.24 \pm 0.32\%$) and C18:2+C18:3 ($37.69 \pm 0.18\%$) constituting 71.93% of total lipids, complemented by $18.79 \pm 0.1\%$ C16:0 as the primary saturated fatty acid (SFA), while MCFA represented $<1\%$. The 25% MLCT2 group showed $1.52 \pm 1.15\%$ C8:0 and $3.91 \pm 0.27\%$ C10:0, whereas 40% MLCT2 contained $0.57 \pm 0.43\%$ C8:0 with elevated C10:0 ($4.88 \pm 2.06\%$). Significant formulation-specific variations emerged in LCFA: the 40% MLCT2 group

demonstrated 10.5% higher polyunsaturated fatty acids (C18:2+C18:3: $37.67 \pm 4.88\%$ vs $31.24 \pm 0.66\%$, $P < 0.05$) concomitant with reduced C18:1 ($32.82 \pm 1.19\%$ vs $36.66 \pm 0.51\%$) and C16:0 ($29.43 \pm 4.24\%$ vs $32.08 \pm 0.67\%$) compared to 25% MLCT2 ($P < 0.05$).

3.7. Basic growth indicators of SD rats

As shown in Fig. 4B-G, after 27 days of feeding, there was no significant difference in body weight, food intake, liver weight, kidney weight, bone weight, and length among the groups. The liver weight of the two MLCT2 groups showed a downward trend, but was not significant.

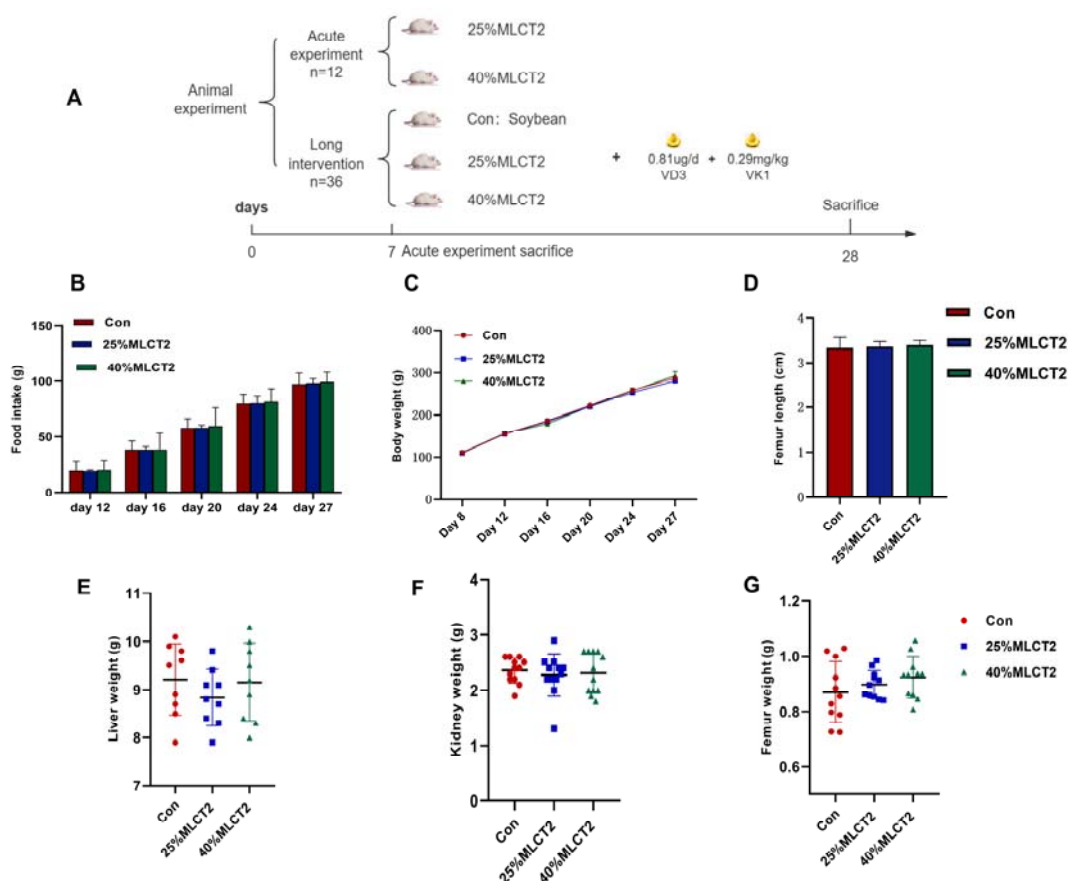


Figure 4. Experimental groups(A) and basic growth indicators of rats(B-G).

3.8. Effect of MLCT composition on Pharmacokinetics of VitD3 and VitK

The pharmacokinetic evaluation revealed concentration-dependent enhancement of vitamin bioavailability through MLCT-mediated delivery (Fig. 5A-D). Both vitamin D3 and K1 exhibited characteristic absorption patterns: serum VitD3 concentrations peaked at 10 h post-administration with complete clearance by 72 h, consistent with previous study³⁰. While VitK1 demonstrated rapid absorption ($T_{max} = 1$ h) and elimination (7 h). Notably, the 40% MLCT2 group significantly elevated C_{max} values for both vitamins (VitD3: 204.6 vs 148.54 $\mu\text{g/L}$; VitK1: 156.08 vs 126.41 $\mu\text{g/L}$, $P < 0.05$) and enhanced systemic exposure as evidenced by AUC differentials (VitD3 AUC0-72h: $38,80.26$ vs $27,91.64$ $\mu\text{g}\cdot\text{h/L}$; VitK1 AUC0-8h: $7,10.12$ vs $5,44.27$ $\mu\text{g}\cdot\text{h/L}$, $P < 0.001$), compared to 25% MLCT2.

Because MCFA and LCFA were released simultaneously during the hydrolysis of MLCT, the

encapsulation and transport of fat-soluble vitamins were primarily mediated by LCFAs. Based on the combined results from *in vitro* digestion and fatty acid analysis in feed, it was observed that the 40% MLCT2 group contained a higher proportion of LCFAs compared to the 25% MLCT2 group. Therefore, the absorption rate of VitD3 and VitK1 in the 40% MLCT2 group was faster, resulting in higher AUC in the 40% MLCT2 group than in the 25% MLCT2 group.

3.9. Effect of MLCT composition on serum vitamin levels

As shown in Fig. 5E, the level of 25OHVD3 was Con (27.3 ± 11.07 ng/mL) < 25% MLCT2 (30.78 ± 13.85 ng/mL) < 40% MLCT2 (34.72 ± 10.85 ng/mL), and 40% MLCT2 group higher control group ($P < 0.01$). In Fig. 5F, the level of VitK1 was Con (22.62 ± 1.97 ng/mL) < 25% MLCT2 (23.36 ± 1.58 ng/mL) < 40% MLCT2 (27.28 ± 2.5 ng/mL) there are significant differences between the experimental group and the control group. These results show that in the long-term intervention trial when MLCT is used as an oil source, 40% MLCT can promote the metabolism of VitD3 and VitK1 more effectively than 25% MLCT, and increase the level of serum 25OHVD3 and VitK1.

3.10. Effect of MLCT on serum biochemical index

As shown in Fig. 5G-H showed that MLCT2 significantly decreased serum TC ($P < 0.05$), in Fig. 5I-K, compared with the control group, 40% MLCT2 reduced serum LDL-C levels while increasing serum HDL-C levels, but there was no significant difference. These results indicated that dietary intake of MLCT has a certain impact on regulating blood lipid metabolism.

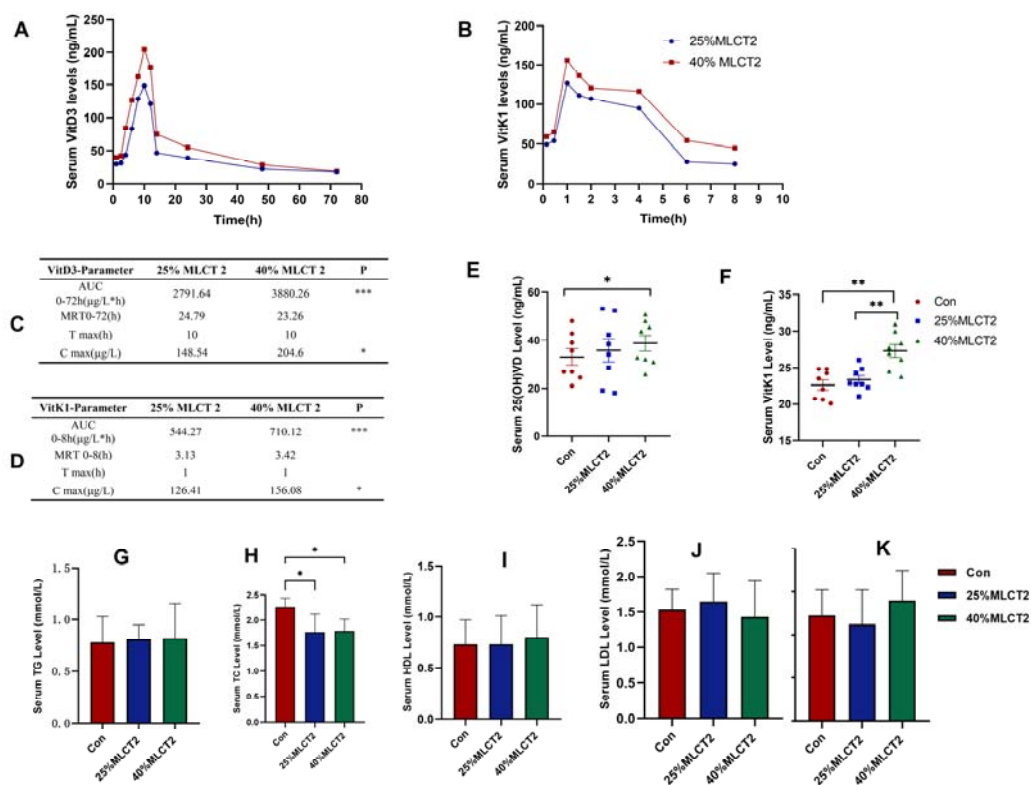


Figure 5. Pharmacokinetic indexes (A-D); serum vitamin content (E-F); blood lipids (G-K).

3.11. Effect of MLCT on bone development in SD rats

Based on the fact that there is no significant difference in bone and femur weight and length between each group. And combined with the serum vitamin levels, the control group and the 25% MLCT2 group are relatively close, therefore, we only compared the 40% MLCT2 group (with higher serum vitamin content) with the control group three-dimensional reconstruction analysis of rat distal femur micro-CT scans.

Compared to the Con group, 66.7% higher BMD in 40% MLCT2 (0.10 ± 0.02 vs 0.06 ± 0.01 g/cm³, $P < 0.001$). MLCT2 (40%) group enhanced trabecular connectivity via increased trabecular number (Tb.N) (1.8-fold), trabecular thickness (Tb.Th) (1.1-fold), and BV/TV (1.9-fold) ($P < 0.05$), while reduced structural heterogeneity evidenced by 42.3% decrease in trabecular separation (Tb.Sp) and 26.9% reduction in trabecular pattern factor (Tb.Pf) ($P < 0.05$) compared to controls (Fig. 6A-J). These microstructural enhancements correlate with MLCT2's optimized vitamin delivery efficiency, suggesting improved bone matrix mineralization through vitamin D/K-dependent osteogenic pathways.

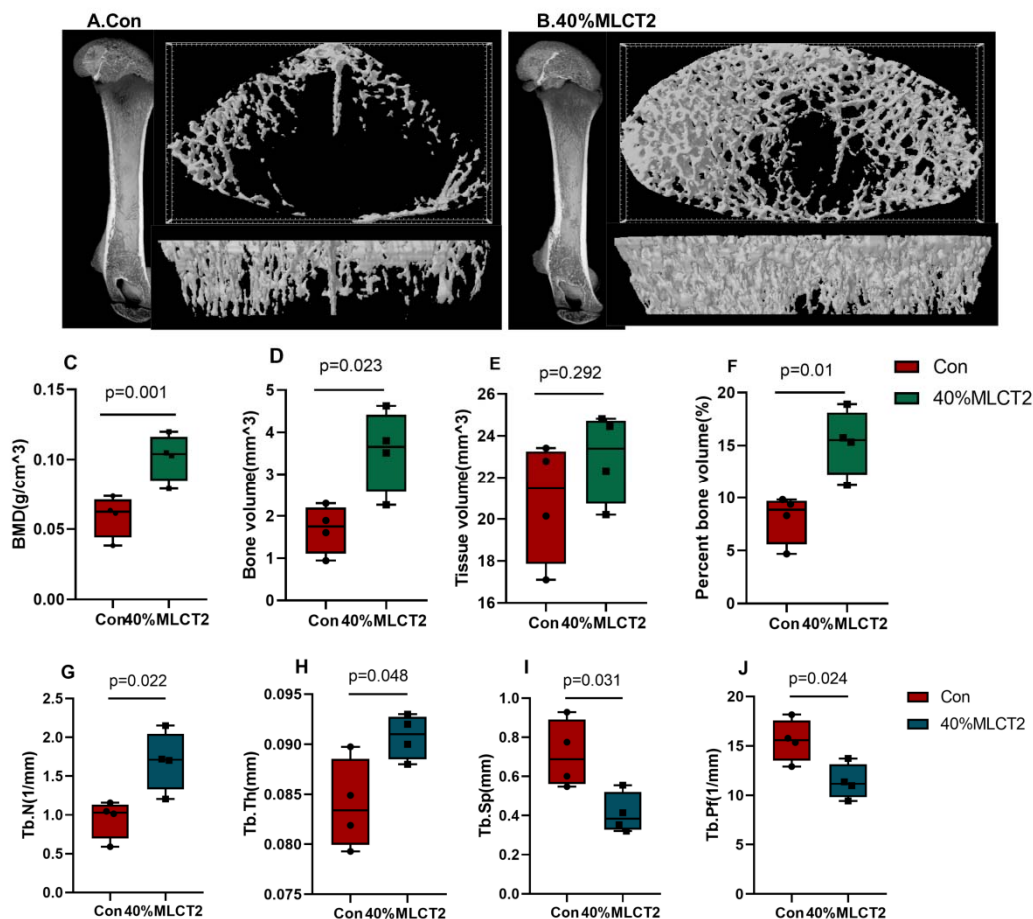


Figure 6. Effects of different fat diets on bone microstructure parameters of SD rats. The representative three-dimensional images of the distal femur of rats were evaluated by μ CT (A-B), BMD (C), and the histomorphometric parameters of healthy bone were measured (D-J).

3.12. Effects of MLCT on intestinal microecology in SD rats

3.12.1. Alpha diversity and OTU of intestinal microecology in SD rats

Based on the results of cluster analysis, the richness of intestinal flora was evaluated by Chao1 and Shannon index (Fig. 7A-B). The Shannon index and Simpson index can reflect the changes in flora diversity, the larger the Shannon index or the smaller the Simpson index, the higher the diversity of flora³¹. The results showed that the order of Chao1 and Shannon index was 40% MLCT2 > 25% MLCT2 > Con. The results showed that the diversity of intestinal flora in the MLCT2 group was richer than that in the control group, but there was no significant difference among different contents of MLCT2. Based on the number of OTUs in each group of samples, the diversity of intestinal flora in the three groups was statistically analyzed by Venn diagram. As can be seen in Fig. 7D, at the bacterial genus level, the three groups had 517 common OTUs. Overall, the number of OTUs in MLCT groups was richer than those in the control group.

3.12.2. Species differences in intestinal flora (phylum level)

This experiment compared the types and abundance of intestinal flora in three groups of SD rats. At the phylum level, the top 10 species with the highest relative abundance were counted, as shown in Fig. 7G, they were mainly: *Firmicutes* (control group was 57.50%; 25% MLCT2 group was 59.48%; 40% MLCT2 group was 63.52%). *Bacteroidetes* (control group was 18.97%; 25% MLCT2 group was 33.94%; 40% MLCT2 group was 32.70%); *Verrucomicrobiota* (control group was 21.53%; 25% MLCT2 group was 3.53%; 40% MLCT2 group was 0.66%). Through the statistical comparative analysis of the level of 7 kinds of bacteria, it is found that there are significant differences among 3 kinds of bacteria, as shown in Fig. 7I. The relative content of *Verrucomicrobiota*, in the control group was significantly higher than that of the 25% MLCT2 group and 40% MLCT2 group. The relative content of *Bacteroidetes*, two MLCT2 groups was significantly higher than that of the control group ($P < 0.05$).

3.12.3. Species differences in intestinal flora (genus level)

The intestinal flora of rats was analyzed at the genus level, and the genera with the top 10 average abundance were selected to draw the stacked column chart (Fig. 7H). For the control group, the main genus was *Akkermansia*, followed by *Alloprevotella*, *Romboutsia*, and *Bacteroides*. For the 25% MLCT2 group, the most important genera were *Romboutsia* and *Alloprevotella*, followed by *Bacteroides*, *Lachnospiraceae_NK4A136_group*, *Clostridium_sensu_stricto_1*. In the 40% MLCT2 group, the most important genus was *Alloprevotella*, followed by *Blautia*, *Bacteroides*, and *Lachnospiraceae_NK4A136_group*.

Through statistical comparison and analysis of 8 common bacterial genera, it was found that there was no significant difference among 5 of the 8 bacterial genera (Fig 7J). Notably, the control group exhibited a significantly higher relative abundance of *Akkermansia* compared to both the 25% and 40% MLCT2 groups ($P < 0.05$). In contrast, *Alloprevotella* levels were markedly elevated in the MLCT2 intervention groups relative to the control ($P < 0.05$). Strikingly, the 40% MLCT2 group demonstrated a profound increase in *Blautia* abundance versus the control cohort ($P < 0.05$). Consistent with phylum-level findings, genus-level analysis of gut microbiota across the three experimental groups revealed critical functional associations.

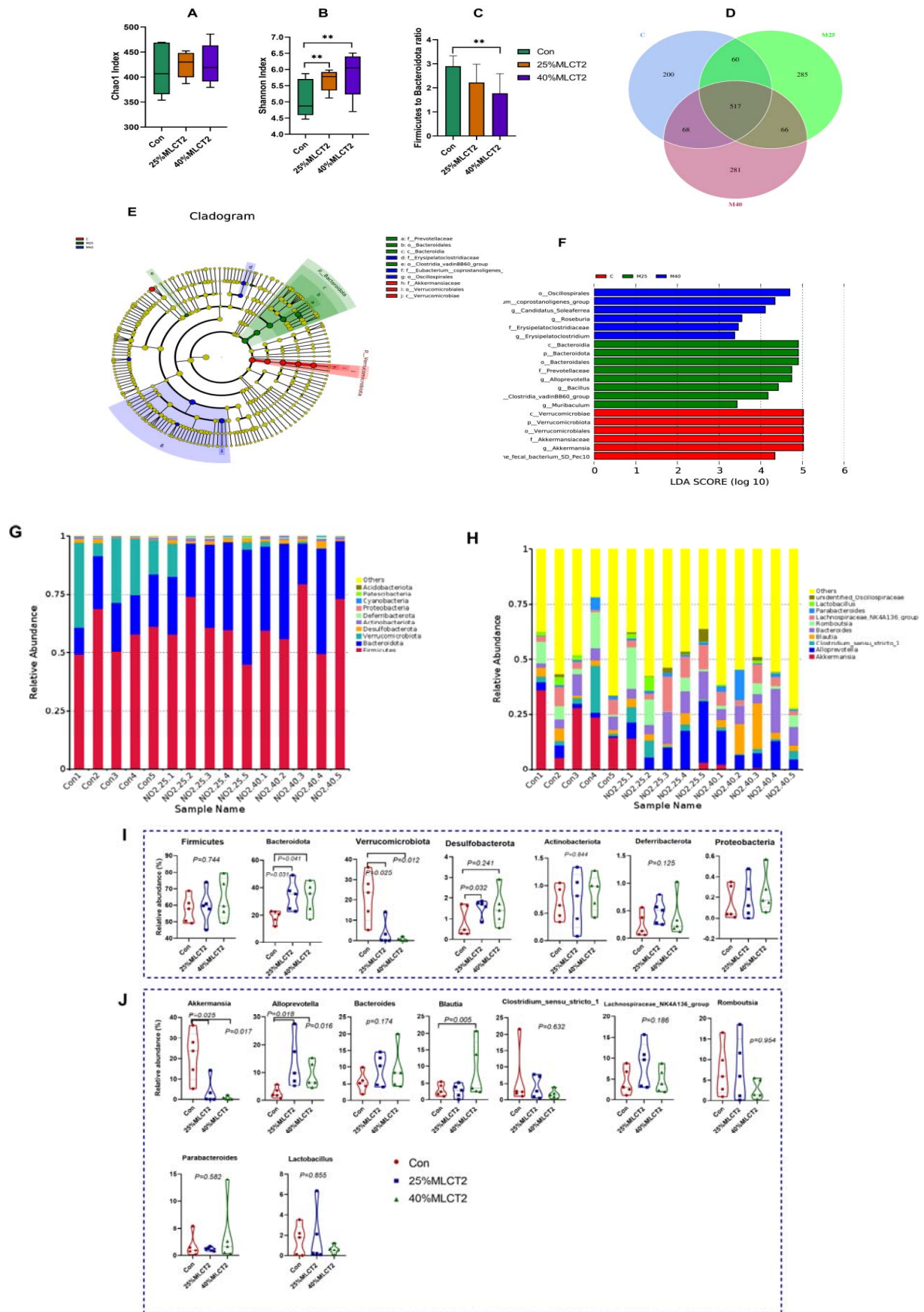


Figure 7. Intestinal flora analysis. α -diversity index (A-C); OTU (D); LEfSe analysis (E-F); differential bacteria at phylum level (G, I) and genus level (H, J).

Note: In G-H, Others represent the sum of the relative abundances of all the other gates except the 10 gates in the figure. Con represents the soybean oil group, NO2.25 represents the 25% MLCT2 group, and NO2.40 represents the 40% MLCT2 group.

3.12.4. Species difference analysis

LEfSe was employed to identify statistically significant biomarkers across experimental groups. As demonstrated in the LEfSe cladogram (Fig. 7E-F), soybean oil-fed SD rats exhibited microbiota signatures dominated by *Verrucomicrobiota* phylum members, specifically class *Verrucomicrobiae*, order *Verrucomicrobiales*, and family *Akkermansiaceae*, with *Akkermansia muciniphila* emerging as the keystone species. In contrast, the 25% MLCT2 group showed enrichment in *Bacteroidales* (order), *Bacillus* (genus), and *Prevotellaceae* (family), while the 40% MLCT2 group harbored predominant populations of *Oscillospirales* (order), *Eubacterium_coprostanoligenes_group*, and *Candidatus_Soleaferrea* (genus).

3.13. Correlation between intestinal flora and VitD3 and VitK1

Spearman's correlation analysis revealed significant associations between gut microbiota composition and MLCT-mediated enhancement of serum 25(OH)VD3 and VitK1 levels (Fig. 8A). 25(OH)VD3 displayed positive correlations with members of the phylum Firmicutes (genus *Lactobacillus*) and phylum Actinobacteria. Conversely, VitK1 exhibited positive associations with Bacteroidetes (genus *Parabacteroides*), Proteobacteria, and genus *Blautia*, while demonstrating inverse relationships with *Akkermansia*, *Romboutsia*, *Lachnospiraceae_NK4A136_group*, and unclassified *Oscillospiraceae* taxa.

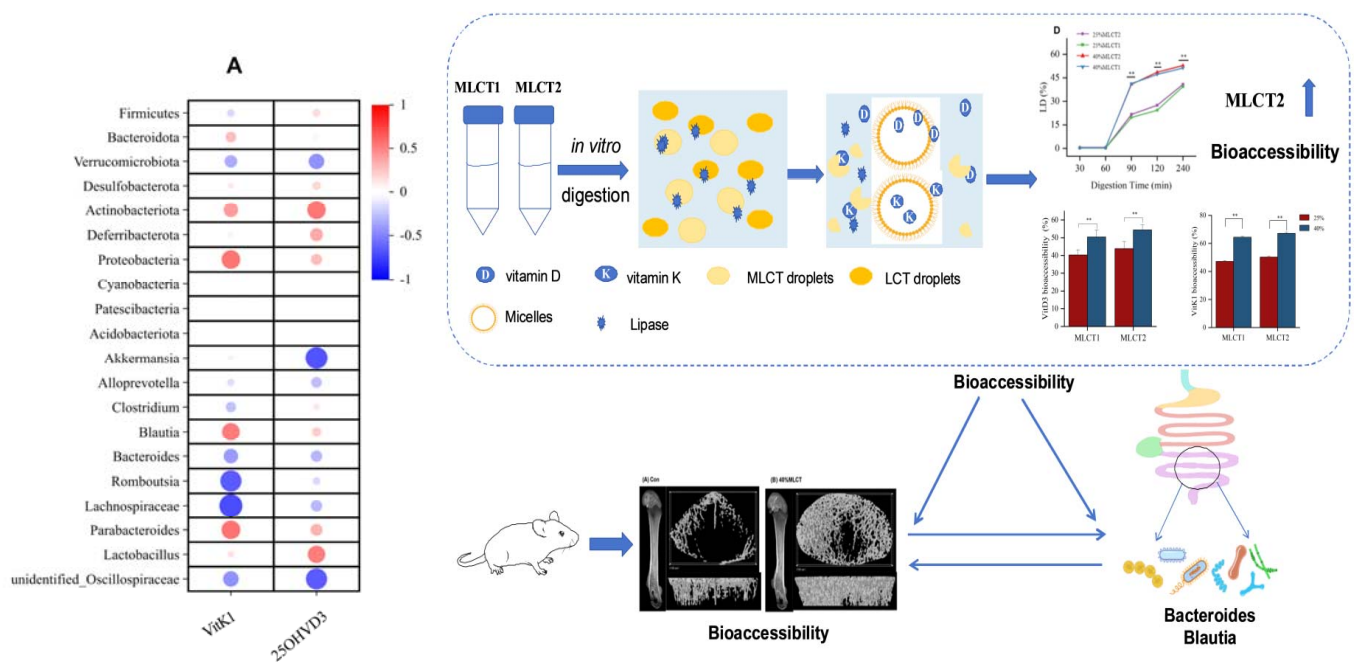


Figure 8. (A) Correlation analysis of gut microbiota with VitD3 and VitK1; (B) MLCT to promote the relationship between VitD3 and VitK1 bioaccessibility and intestinal microflora.

4. Discussion

4.1 Higher MLCT concentration (40%) enhances vitamin D/K bioavailability via improved hydrolysis and micellar solubilization

To investigate the dose-dependent effects of varying MLCT concentrations (ranging from 25% to 40%) in human milk on VitD and VitK bioavailability, we conducted comprehensive *in vitro* and *in vivo* studies. The *in vitro* digestion experiments demonstrated significantly higher bioaccessibility of VitD3 and VitK1 in

the 40% MLCT group compared to the 25% MLCT group ($P < 0.05$). Crucially, a comparative analysis between MLCT1 and MLCT2 at the same concentration (40%) revealed that MLCT2 consistently outperformed MLCT1 in enhancing vitamin bioaccessibility, indicating that not only the quantity but also the specific structural composition of MLCT is pivotal.

The superior performance of MLCT2 can be mechanistically attributed to its distinct TAG architecture and resultant fatty acid profile, which collectively promote more efficient hydrolysis and the formation of mixed micelles with enhanced solubilization capacity for lipophilic vitamins. Our compositional analysis revealed fundamental structural divergences between MLCT1 and MLCT2. MLCT2 possessed a significantly higher proportion of MLL type TAGs (40.48% vs. 36.56% in MLCT1) and a correspondingly lower proportion of MML type TAGs (21.25% vs. 27.51% in MLCT1) (Fig. 1B). This structural shift was accompanied by a marked enrichment in specific PUFA-containing TAG species in MLCT2, such as Ca/L/L and Ca/O/L (Table S1). Consequently, the overall fatty acid profile of MLCT2 was characterized by a 9.9% higher content of C18:2+C18:3 PUFA (49.06% vs. 39.12% in MLCT1, $P < 0.05$) and a 4.9% reduction in C16:0 (Table S2).

This unique structure directly translates to enhanced hydrolysis kinetics. The MLL-rich structure of MLCT2, where long-chain fatty acids (LCFAs, predominantly PUFAs) occupy two positions on the glycerol backbone, allows pancreatic lipase (with its sn-1,3 regiospecificity) to sequentially hydrolyze two LCFAs per TAG molecule. This process efficiently generates two molecules of long-chain free fatty acids (FFAs) and one molecule of medium-chain monoacylglycerol (MAG). In contrast, the higher MML content in MLCT1 leads to the release of more medium-chain fatty acids (MCFAs) and a long-chain MAG upon complete hydrolysis. The higher proportion of LCFAs/PUFAs released from MLCT2 is critical, as LCFAs, particularly PUFAs with their kinked chains, contribute more effectively to the formation and stabilization of mixed micelles with larger hydrophobic cores compared to MCFAs^{14, 32}. This was corroborated by our *in vitro* digestion data, which showed that 40% MLCT2 achieved the highest final FFA release (52.81%, $P < 0.05$) and yielded digestion products with a significantly higher proportion of C18:2 (56.84%) compared to other groups (Fig. 2D, 3).

Furthermore, the improved hydrolysis efficiency of MLCT2's specific TAG structure limits the accumulation of undigested lipolysis-resistant species. Post-digestion lipidomic analysis revealed that the 40% MLCT2 group had a lower residual TG content (4.52%) and a significantly reduced retention of MLL-type TGs (9.78%) compared to the 25% MLCT2 group (6.63% and 18.29%, respectively) (Fig. 3C, D; Table S3). This indicates that the MLL-rich structure of MLCT2 is more accessible and susceptible to lipase action, preventing the persistence of poorly hydrolyzed TAGs that could otherwise sequester fat-soluble vitamins. The residual undigested TGs across all groups were predominantly long-chain LLL-types (e.g., TG 18:1/18:2/18:2), which are known to be hydrolyzed more slowly, further highlighting the metabolic advantage of the MLCT structure¹⁶. It should be noted that a limitation of this study is that the prepared emulsion droplets ($D_{4,3} \approx 200\text{--}660\text{ nm}$) were relatively small compared to the fat globules in human milk, which may affect the

comparative analysis of lipid digestion kinetics with physiological conditions. Therefore, the findings of this study are more suitable for revealing mechanistic trends rather than directly quantifying the actual digestion process of human milk in infants.

In summary, the superiority of MLCT2 stems from a synergistic interplay of its structural attributes: 1) A higher MLL-type TAG content facilitates efficient lipolysis and maximizes the release of LCFAs/PUFAs; 2) The enriched PUFA profile (C18:2/C18:3) of these LCFAs promotes the formation of mixed micelles with expanded hydrophobic domains, thereby enhancing the solubilization capacity for VitD3 and VitK1¹⁴; and 3) This optimized structure minimizes the formation of hydrolysis-resistant TAG fragments, ensuring a greater proportion of vitamins are liberated and incorporated into the bioaccessible micellar phase. This structure-function relationship provides a clear mechanistic basis for the observed enhancement in vitamin bioavailability.

4.2 40% MLCT2 outperforms 25% in promoting vitamin D/K bioavailability

Building upon *in vitro* infant digestion models demonstrating superior bioaccessibility enhancement of fat-soluble vitamins by 40% MLCT2 group versus 25% MLCT2 group ($P < 0.05$), we further validated these findings through *in vivo* animal studies. Pharmacokinetic analyses demonstrated significantly elevated C_{max} and AUC values for VitD3 and VitK1 in the 40% MLCT2 group compared to the 25% MLCT2 group ($P < 0.05$). Chronic animal studies further corroborated this enhanced absorption profile, with the 40% MLCT2 group exhibiting higher serum 25OHVD and VitK levels, than the 25% MLCT2 group ($P < 0.01$). These findings collectively indicate that MLCT2 matrices accelerate the absorption kinetics of lipophilic vitamins, with bioenhancement effects proportional to MLCT concentration gradients.

Compared to LCFAs, MCFAs exhibit superior aqueous dispersibility, enabling their rapid removal from droplet surfaces. This property facilitates sustained lipase-mediated hydrolysis³⁰. In contrast, LCFAs tend to accumulate at droplet interfaces, thereby inhibiting further lipolytic activity. These findings suggest that structured lipids such as MLCT promote more efficient enzymatic lipid digestion compared to simple physical mixtures of MCT/LCT. Yang et al.³³ reported that the bioavailability of lipophilic compounds in LCT was significantly higher than in MCT, likely because LCFAs generate substantially larger hydrophobic domains than MCFAs, allowing greater incorporation of lipophilic vitamins and hydrophobic compounds.

Pharmacokinetic analyses (including AUC values) or serum concentrations of fat-soluble vitamins, consistently demonstrated higher values in the 40% MLCT2 group than in the 25% MLCT2 group. This indicates that the 40% MLCT2 group released more MCFAs and LCFAs, thereby accelerating the absorption of VitD3 and VitK1. Furthermore, MLCTs play a critical role in infant nutrition due to their moderate digestion and absorption rates, which fit the immature intestinal tract of infants.

4.3 MLCT indirectly promotes bone Health by enhancing VitD3/VitK1 absorption

During lipid digestion, triacylglycerols undergo enzymatic hydrolysis by pancreatic lipase and bile salts, yielding MAGs and FFAs that subsequently assemble with bile salts into mixed micelles, which facilitate the solubilization and transport of lipophilic vitamins (e.g., VitD3 and VitK1) and other lipid-soluble nutrients

across the intestinal epithelium. Critically, lipid matrices enhance micellar solubilization capacity by expanding the hydrophobic core volume, thereby significantly improving vitamin dissolution efficiency and transepithelial absorption rates¹⁶.

In vitro digestion models demonstrated that the 40% MLCT2 group exhibited 1.24-fold and 1.34-fold higher bioaccessibility of VitD3 and VitK1 compared to 25% MLCT2 group ($P < 0.05$), with residual undigested triglycerides predominantly identified as long-chain LLL-type species (e.g., TG 18:2/18:2/18:2), confirming MLCTs' heightened susceptibility to lipase-mediated hydrolysis. *In vivo* animal studies revealed that MLCT-enriched diets elevated serum 25(OH)VD levels by 7.46 ng/mL ($P < 0.01$) and increased BMD by 1.67-fold relative to soybean oil controls ($P < 0.001$). Micro-CT analysis of SD rats further demonstrated that the 40% MLCT2 group exhibited significant improvements in trabecular bone microarchitecture, including higher Tb.N, Tb.Th, and increased BV/TV, alongside reductions in Tb.Sp and Tb.Pf (all $P < 0.05$ vs. controls). These findings mechanistically link MLCTs' optimized lipid matrix to enhanced VitD3 and VitK1 bioavailability, thereby promoting skeletal development and mitigating fracture risks through improved calcium deposition and osteocalcin activation.

As is well known, VitD3 has emerged as a well-established agent for the prevention of osteoporosis. A recent meta-analysis conducted among individuals aged 65 and older has confirmed that a VitD3 supplementation of at least 20 µg/d can significantly increase bone density and reduce the risk of fractures³⁴⁻³⁶. It is worth noting that a wealth of current studies have reported on the role of VitK2 in bone remodeling and skeletal health³⁷. Zhou et al.³⁸ reported the results of a meta-analysis of nine randomized controlled trials, which confirmed that VitK2 plays a crucial role in maintaining bone mineral density. VitD and VitK play pivotal roles in infant skeletal development. Research reveals that in 6-month-old infants, regardless of vitamin AD drop supplementation, the BMD of the breastfed group is considerably higher than that of the formula-fed group, but significantly lower than the mixed-fed group³⁹. This indicates that enhancing VitD and VitK delivery systems can boost infants' absorption and utilization of VitD3 and VitK1. It further demonstrates that MLCT synthesized by transesterification has a wide range of application significance as a new fat-soluble vitamin carrier for promoting its bioavailability.

4.4. MLCT boosts vitamin D/K uptake via micellar transport and microbiota-driven nutrient recycling

In the study of intestinal microflora, it is found that MLCT has a great influence on the overall structure of intestinal microflora, and the degree of influence depends on the proportion of MLCT. A study has reported that MLCT can reduce the ratio of F/B⁴⁰, and our research was consistent with that. As expected, the ratio of F/B in the MLCT2 group was significantly lower than that in the control group (Fig. 7C) (Con > 25% MLCT2 > 40% MLCT2). The results showed that the feed composed of MLCT2 with more MLCT could significantly reduce the ratio of F/B in the intestinal tract of SD rats. Yue et al.⁴¹ found that MLM-type MLCT structural lipids can regulate the bacterial composition of *ApoE* (-1) mice, reduce the proportion of *Bacteroides*, and increase the relative abundance of short-chain fatty acid-producing bacteria. In our study, there were significant differences in microflora structure (α diversity) between the MLCT2 group and the control group.

Therefore, we hypothesize that the impact of MLCT2 on gut microbiota could be attributed to its predominant MML/MLL configuration. When digested, these structured triglycerides generate micelles richer in LCFA, thereby encapsulating higher levels of VitD3 and VitK1 compared to conventional triglycerides. In contrast, soybean oil triglycerides primarily composed of LLL configuration exhibit slower lipolysis kinetics than their MML/MLL counterparts, potentially resulting in reduced micelle formation efficiency and subsequent nutrient bioavailability, ultimately influencing gut microbial diversity.

MCFA exhibits potent bacteriostatic effects, they can enhance intestinal morphology, modulate gut microbiota structure, suppress harmful microbial colonization, and promote gut health⁴². Compared to the soybean oil diet, the MLCT2 diet contained higher levels of medium-chain fatty acids (MCFAs), which contributed to the observed differences in their gut microbiota. This suggests that a diet with a specific fatty acid profile (MCFA+LCFA) results in a richer microbiota than a diet based on a single source (soybean oil). Gut microbiota and micronutrients interact bidirectionally⁴³. Vitamins can alter gut microbiota composition by influencing immunity, bacterial growth, and metabolism. For instance, dietary VitD supplementation in infants shapes early microbial composition, lowers the incidence of neonatal digestive diseases, and prevents neonatal calcium deficiency⁴⁴. Conversely, VitD deficiency in children reduces bacterial diversity (Barone et al., 2022). Early VitD supplementation improves children's gut microbiota and function, prevents obesity, and supports healthy growth (Xiaoli, 2022). Moreover, neonatal VitD supplementation is influenced by gut microbiota, which also affects the synthesis, metabolism, and transport of fat-soluble vitamins and their metabolites⁴⁵⁻⁴⁹. Animal and human studies show gut microbiota can synthesize VitK, VC, and B vitamins, benefiting gut health⁵⁰.

The *Akkermansia* genus (phylum Verrucomicrobiota) exhibited significantly elevated abundance in the Con group, which may drive enhanced lipid metabolism as evidenced by reduced TG and TC levels in SD rats. Conversely, *Alloprevotella* (phylum Bacteroidetes) demonstrated marked enrichment in the MLCT2 intervention groups ($P < 0.05$), potentially promoting VitK biosynthesis. This observation aligns with the established role of *Bacteroides* species as primary VitK producers in the human gut, a metabolic pathway implicated in improved bone mineral density and therapeutic strategies against osteoporosis⁴³.

LEfSe and Spearman correlation analyses revealed distinct microbial signatures across experimental groups. The control group exhibited *Verrucomicrobiota* dominance (phylum level), whereas the 25% MLCT2 group was enriched with the order *Bacteroidales* and family *Prevotrellaceae*. Notably, the 40% MLCT2 group harbored unique taxa including order *Oscillospirales* and the *Eubacterium_coprostanoligenes_group* (genus level). Zhu et al.⁵¹ demonstrated that VitD deficiency correlates with microbiota-driven reductions in mucin layer integrity, characterized by elevated *Akkermansia muciniphila* – a mucin-degrading bacteria. This mechanistic link aligns with our observations: the soybean oil group displayed both higher *Akk* abundance and significantly lower serum 25-hydroxyvitamin D levels compared to the MLCT2 group ($P < 0.05$).

Mechanistically, *Bacteroides*–recognized as primary VitK producers in the human gut–demonstrated a positive correlation with serum VitK1 levels in MLCT2 groups (Fig. 8A). This aligns with clinical

observations: infants with elevated *Bacteroides* abundance exhibit enhanced neurodevelopmental outcomes⁵², while VitD supplementation increases *Bacteroides fragilis* and *Prevotellaceae* populations in human cohorts⁵³. The 40% MLCT2 group's unique microbiome profile, characterized by *Oscillospirales* and *Eubacterium* enrichment, may drive differential metabolic effects. Critically, *Eubacterium* species are known butyrate producers with demonstrated immunomodulatory functions, suggesting their potential role in MLCT2-mediated nutrient absorption enhancement. It should be noted that although the SD rat is a valuable animal model, there are fundamental differences in its gut microbiota and physiological characteristics compared to human infants.

We speculate that the enhanced bioavailability of VitD3 and VitK1 mediated by transesterified MLCT stems from its unique triacylglycerol architecture. Mechanistically, MML/MLL-structured MLCT preferentially forms micelles than MCT and LCT during intestinal digestion, effectively encapsulating fat-soluble vitamins (Fig. 8B). This process stimulates the expression of scavenger receptor class B type I (SR-BI) and Niemann-Pick C1-like 1 (NPC1L1) transporters in enterocytes⁵⁴, thereby facilitating systemic absorption of VitD3 and VitK1. Concurrently, MLCT2 modulated gut microbiota composition, elevating beneficial taxa including Firmicutes (notably genus *Blautia*), Bacteroidetes, and *Alloprevotella*, while suppressing the sulfate-reducing pathogen *Desulfobacterota*. Crucially, bidirectional interactions between microbial communities and fat-soluble vitamins were observed: (1) MLCT-enhanced VitD3/VitK1 bioavailability promoted probiotic growth; (2) Undigested vitamins reaching the colon underwent microbial reactivation via *Blautia*-mediated metabolic pathways, establishing a positive feedback loop that further amplified nutrient absorption efficiency (Fig. 8B).

These findings collectively suggest that MLCT exerts dual synergistic effects - direct physicochemical enhancement of vitamin solubilization coupled with microbiota-driven metabolic recycling - ultimately amplifying host nutrient utilization and intestinal homeostasis.

Declaration of interests

The authors declare no competing interest.

Acknowledgements

This study was supported by Jiangxi Special Project for Fiscal Science and Technology, Nanchang University (ZBG20230418011), Key Research and Development Program of Jiangxi Province (20223BBF64002), National Key Research and Development Program of China (2023YFF1104502), National Center of Technology Innovation for Dairy (2022-Ke Yan Gong Guan-4).

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